Molecular ecology and social evolution of the eastern carpenter bee,

Xylocopa virginica

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Abstract

Bees are extremely valuable models in both ecology and evolutionary biology. Their link to agriculture and sensitivity to climate change make them an excellent group to examine how anthropogenic disturbance can affect how genes flow through populations. In addition, many bees demonstrate behavioural flexibility, making certain species excellent models with which to study the evolution of social groups. This thesis studies the molecular ecology and social evolution of one such bee, the eastern carpenter bee, *Xylocopa virginica.* As a generalist native pollinator that nests almost exclusively in milled lumber, anthropogenic disturbance and climate change have the power to drastically alter how genes flow through eastern carpenter bee populations. In addition, *X. virginica* is facultatively social and is an excellent organism to examine how species evolve from solitary to group living.

Across their range of eastern North America, *X. virginica* appears to be structured into three main subpopulations: a northern group, a western group and a core group. Population genetic analyses suggest that the northern and potentially the western group represent recent range expansions. Climate data also suggest that summer and winter temperatures describe a significant amount of the genetic differentiation seen across their range. Taken together, this suggests that climate warming may have allowed eastern carpenter bees to expand their range northward. Despite nesting predominantly in disturbed areas, eastern carpenter bees have adapted to newly available habitat and appear to be thriving. This is in marked contrast to many other bee species, particularly in the genus *Bombus*, which appear unable to shift their ranges along with climate change.

Facultatively social organisms are excellent species to study the evolution of social groups, and the remaining chapters address questions of sociality in *X. virginica.* I used observation nests and genetic relatedness to examined how females behave towards one another in the spring prior to the establishment of dominance hierarchies in social nests. In spring, females directed fewer aggressive behaviours and more cooperative behaviours towards familiar rather than related individuals, indicating that females use nestmate recognition rather that kin recognition when interacting with conspecifics. Overwintering groups often contain both related and unrelated individuals, indicating that many bees interacting with one another in the fall prior to overwintering may be unrelated, emphasizing the importance of recognizing nestmates.

Within social carpenter bee nests three different types of female have been described: primary, secondary and tertiary. Primary females are the dominant foragers and egg layers in the nest while secondary and tertiary females appear to join a reproductive queue behind the primary. To understand the nature and flexibility of this reproductive queue I performed removal experiments across three different years. This study showed that secondary females always assumed the role of replacement primary, while tertiary females rarely opted to forage and reproduce even if they were the only female in the nest. Removal experiments demonstrated that social groups in *X. virginica* are complex and comprise two different reproductive strategies (breed in the current year or delay reproduction) as well as form dominance hierarchies among primary and secondary females. Several tertiary females were able to become primary or solitary females in their second summer, providing evidence for how each type of female may have evolved in social nests.

Finally, I examined how competition influences the evolution and maintenance of social groups in eastern carpenter bees. In conditions of high population density significantly more social nests were present in the population, indicating that competition for limiting nesting resources drives individuals together into social groups. Within social groups relatedness was low, and siblings actually dispersed away from one another to other nests in the population, reducing competition among kin. Eastern carpenter bees appear to demonstrate an interesting evolutionary route to sociality, where very high levels of competition among kin lead to dispersal, while limited nesting substrate forces individuals back into unrelated social groups. While predicted by kin selection, social groups of this nature are previously undescribed in the Hymenoptera, and further study of eastern carpenter bees can provide novel insights into alternate routes to sociality.

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Chapter 1: General Introduction

This thesis is about the molecular ecology and social evolution of the eastern carpenter bee *Xylocopa virginica* (Linnaeus 1771). Females of this species can be found nesting both solitarily and in groups, making them an excellent species with which to study the evolution of sociality, one of the most interesting questions in evolutionary biology today. Eastern carpenter bees also nest primarily in milled lumber, linking them to anthropogenic disturbance across their range. In the face of global pollinator decline, elucidating the population genetic structure of a generalist pollinator living in disturbed environments will provide valuable information on how native pollinators are affected by anthropogenic change. The research presented in this thesis can be subdivided into two main themes: molecular ecology and social evolution. In chapter two I use microsatellite markers to describe the population genetic structure of *Xylocopa virginica* across eastern North America. Chapters three through five explore the nature of social groups in *X. virginica* using behaviour, experimental manipulations, genetics and detailed observations to understand how decisions are made within social groups and how sociality has evolved in this remarkable species.

Molecular ecology

Ecology is defined as the study of how organisms interact with their environment (Molles 2005). Molecular ecology incorporates techniques from molecular biology to answer ecological questions from new perspectives (Beebee & Rowe 2008). The impact of molecular techniques to the field of ecology has been broad and significant. For example, DNA sequence data have been useful in delineating new and cryptic species

(Hebert et al. 2004; Barrett & Hebert 2004; Kuhlmann et al. 2007; Vickruck et al. 2011), and population genetic techniques have been employed when assessing the conservation status of a species (Packer et al. 2005; Dixon et al. 2007). Genetic data are also a useful tool to infer relationships among groups, and allows for the inference of relatedness without having detailed pedigrees (Queller & Goodnight 1989; Dugatkin 2014). This thesis uses molecular ecology techniques to understand both population structure of *X. virginica* across its range as well as to calculate genetic relatedness among individuals in social groups.

Population genetic parameters

In order to assess the genetic variation among populations, the amount of variation must first be quantified. One common method of inferring genetic variation is through the use of microsatellites. A microsatellite is a hypervariable region of DNA typically found in non-coding regions of the genome (Tautz 1989). Mutations within a microsatellite locus are most often neutral, allowing for multiple alleles to persist at each locus. Microsatellite loci are typically 200-500 base pairs in length and are amplified using locus specific primers and polymerase-chain reactions. Once the locus has been amplified DNA fragment lengths are quantified to assign alleles (Beebee & Rowe 2008). Genotyping multiple microsatellite loci within a single individual serves as a representative sample of genetic diversity across the genome, which can then be compared to other individuals within the population and across their range.

Assessing population genetic parameters involves quantifying allelic variation within populations as well as among them. To do this, many individuals are sampled

from multiple populations across the geographic range of interest. Comparing allelic variation per population, as well as how the proportions of alleles differ among populations allows for the inference of many population genetic parameters, including levels of observed and expected heterozygosity, inbreeding, and effective population size. Information generated from microsatellite loci can also be used to compare genetic differentiation among populations.

Population genetic parameters within and among populations

Within populations, several indices can be calculated to provide information about individual groups. For example, observed levels of heterozygosity can be compared to expected levels of heterozygosity under Hardy-Weinberg equilibrium. Significant deviations from the expected values can have biological implications such as inbreeding or limited genetic exchange between the groups (Beebee & Rowe 2008). Increased levels of inbreeding can lead to increased homozygosity and accumulation of deleterious alleles (Keller & Waller 2002). Allelic richness is defined as the mean number of alleles per locus. Reductions in allelic richness can be used to infer range expansion or population isolation, which often reduces the number of alleles in the affected population(s) as compared to the rest of the dataset (Excoffier et al. 2009; Garroway et al. 2011). Effective population size (Ne), represents the number of breeding individuals in the population, as opposed to the number of sampled individuals. Effective population size is a particularly important parameter when studying organisms that are long lived, take several generations to become reproductive, or when only few member of a large group reproduce (such as in many species of social insect; Packer & Owen 2001; Zayed 2004).

Quantifying differences among populations also provides important information about how genes move through populations. Pairwise comparisons, such as $F_{ST,}$ allow for the delineation of how the proportions of alleles at each locus differ between populations (Wright 1965). In theory F_{ST} varies between zero and one. When $F_{ST} = 0$ the two populations share the same alleles in the same proportions across every locus. When $F_{ST} = 1$, the two populations do not share a single allele at any locus, indicating that there is no gene flow among these two groups (Wright 1965). In the last twenty years several other statistics have been developed to quantify genetic differentiation between populations (e.g. D_{EST}, G_{ST}), but all with the same aim to describe population differentiation (Jost 2008; Whitlock 2011). Other methods also exist to quantify differentiation among populations. Analysis of Molecular Variance (AMOVA) examines how genetic variation is partitioned among and within groups, and significant differentiation between groups indicates population structuring (Excoffier et al. 1992). Finally, software employing Bayesian assignment tests can be used to group populations into distinct genetic clusters, defining where substructuring occurs across the range of a species (Pritchard et al. 2000; Guillot et al. 2005). These powerful, computer intensive methods can be used without incorporating knowledge of sample location, such as in the program Stucture (Pritchard et al. 2000) or including geographic information as accomplished by Geneland (Guillot et al. 2005). Elucidating where substructuring takes place across the range of a species can provide clues as to where barriers to gene flow occur.

Landscape and environmental factors affecting gene flow across species distributions

Several factors can influence how and where individuals are able to move and reproduce within the landscape, often creating groups of populations which are genetically distinct from others (Darvill et al. 2006; Latch et al. 2011; Wellenreuther et al. 2011 for examples). Some features, such as those found across the landscape, are natural. Other factors, such as habitat fragmentation and climate change, are created by anthropogenic disturbance. Natural landscape features, such as large bodies of water or mountain ranges, can limit dispersal, therefore limiting gene flow among populations on either side of the geographic barrier, increasing the amount of genetic differentiation among populations on either side (Church et al. 2003; Soltis et al. 2006; Davis et al. 2010; Norén et al. 2011). Perhaps one of the most well known examples of a landscape feature causing genetic differentiation is in the salamander, *Ensatina eschscholtzii,* in which limited gene flow across a mountain range led to the formation of seven different subspecies (Moritz et al. 1992).

Anthropogenic disturbance resulting in habitat fragmentation can also lead to genetic differentiation among populations. By removing suitable habitat, fragmented patches of the remaining landscape can make dispersal between patches difficult or impossible, isolating populations from one another and reducing the levels of gene flow. These smaller, less connected patches are more susceptible to genetic drift and typically have lower genetic variation that can be used to adapt to local conditions (Ellis et al. 2006; Lozier et al. 2011). The process of habitat fragmentation leading to higher levels of genetic differentiation among populations and subsequently lower genetic diversity has

been documented across many taxa from invertebrates (Keller & Largiadèr 2003; Darvill et al. 2006, 2010; Ellis et al. 2006) to vertebrates (Noël et al. 2007; Dixo et al. 2009; Haag et al. 2010).

Anthropogenic disturbance leading to rising global temperatures have also been shown to alter areas of suitable landscape available to species, causing range shifts, range contractions, or discontinuity between patches of suitable habitats (Chen et al. 2011; Kuhlmann et al. 2012; Pomara et al. 2014; Kerr et al. 2015). In order for a species to take advantage of a shifting range, it must expand into previously un-colonized territory. These range shifts are often detectable through population genetic parameters (Excoffier et al. 2009; Hoglund 2009). Populations at the front of the expansion may undergo bottlenecks, show reduced allelic diversity, and have smaller effective population sizes which in turn can lead to reduced levels of gene flow and higher levels of population genetic structure (Excoffier et al. 2009).

Population genetic structure in bee communities

Recent studies have shown that native bees may be particularly influenced by anthropogenic disturbance (Darvill et al. 2006; Goulson et al. 2015; Kerr et al. 2015). Habitat destruction is causing increased fragmentation among populations, and climate change is causing the range of many bee species to shift (Dellicour et al. 2015a; Kerr et al. 2015). While the importance of native pollinators is increasingly obvious, we have limited knowledge of how native species, particularly those tightly linked to human colonization, respond to anthropogenic disturbance. Molecular ecology tools offer an excellent way to empirically examine the genetic composition of important pollinator

species, to quantify both genetic variability within populations across the range of a species, and to identify populations that may be at risk due to small effective population sizes or inbreeding due to reduced gene flow.

A very recent study on the squash bee, *Peponapis pruinosa*, demonstrated that its role as a specialist pollinator of squash facilitated a rapid, agriculturally induced range expansion across North America (López-Uribe et al. 2016). To increase our understanding of how native bee species are affected by human disturbance, more studies like that of *P. pruinosa* are needed. Understanding how generalist pollinators are affected by disturbance will greatly enhance our knowledge of how native pollinators adapt to anthropogenic change. Prime candidates for these types of population genetic studies are bees with broad geographic ranges linked to human colonization, such as the eastern carpenter bee, *Xylocopa virginica*.

Social evolution

The question of how social groups arise and why non-reproductive individuals remain in the nest to help other individuals raise offspring, has been a persistent question in evolutionary biology since Darwin described natural selection (Bourke 2011a). The second main aim of this thesis is to understand how social groups form and are maintained in the eastern carpenter bee *Xylocopa virginica*. To do this, I have focussed on three smaller objectives. First, I ask how individual recognition functions in carpenter bee groups. How the members of a group recognize one another has consequences for group stability and membership. How individuals recognize and behave towards one another also has implications for the evolution of dominance hierarchies and reproductive

strategies in social nests, which I explore in chapter four of this thesis. Finally, I ask what ultimate factors are involved in the evolution of social groups in *X. virginica*, focussing on the role that kin competition plays in group formation and composition.

Using molecular ecology to infer kinship patterns

Molecular ecology techniques can also be used to infer genetic relatedness among individuals in family groups. In this context the term relatedness refers to the proportion of genes identical by descent that are shared by two individuals. Relatedness is typically represented by *r*, which can vary from zero (individuals share no genes identical by descent) to one (individuals share all alleles at every locus). Calculating values of relatedness allows for the quantification of indirect fitness, or fitness attained through the successful reproduction of relatives who share genes with the focal individual, which are identical by descent. For example, diploid siblings share on average one half of their genes, therefore $r = 0.5$ (Dugatkin 2014). One method of inferring relatedness is by using neutral genetic markers such as microsatellites. Microsatellites are co-dominant genetic markers, meaning that homozygotes and heterozygotes can be distinguished from one another.

When inferring relatedness using allelic variation at microsatellite loci, it is important to take into account how common each allele is in the population. Alleles that are very common may be shared by individuals by chance as opposed to being identical by descent. Queller and Goodnight (1989) developed a method to calculate relatedness while accounting for allele frequencies:

$$r = (\sum_i \sum_l \sum_a (P_y - P)) / (\sum_i \sum_l \sum_a (P_x - P))$$

where P is the frequency of any allele, P_x is the frequency of that allele in the focal individual's genome and P_y is the frequency of that allele in the other individual's genome. This value is summed over alleles (a), loci (l) and individuals (i; Queller & Goodnight 1989). By calculating relatedness among group members I will be able to quantify the role that indirect fitness plays in social group formation in *X. virginica.*

How do individuals in social groups recognize one another?

Recognition is the ability of one individual to consistently differentiate one another (Sherman et al. 1997). This ability is important in many contexts, such as inbreeding avoidance (Pusey & Wolf 1996), prey avoidance when sibling cannibalism can occur (Pfennig et al. 1993), neighbour recognition (Brindley 1991) and social group cohesion (Breed 2014). Two primary types of recognition have been recognized in social groups: kin recognition, which is the ability to recognize conspecifics to which an individual is related, and non-kin based recognition, where individuals can recognize one another based on non-genetic traits. Non-kin recognition can be further subdivided into group member recognition and individual recognition (Breed 2014). Non-kin based recognition can take place among kin and non-kin (kin can be recognized as group members or individuals). Simply put, kin recognition states that conspecifics identify one another based on genetic relatedness. This implies that phenotypic traits are an indicator of genetic relatedness and that conspecifics can detect how many of these traits are identical by descent (Lacy & Sherman 1983). Group or individual recognition implies that phenotypes are learned and remembered during subsequent interactions. There are several mechanisms by which recognition can take place, some which apply only to kin

selection, some to group/individual recognition and some to both. These include green beard effects, phenotype matching, self referencing and learned familiarity.

For recognition to occur through self-referencing, individuals have to perceive their own phenotype and then use that knowledge to make decisions as to how much of the phenotype of a conspecific matches their own (Breed 2014). Self-referencing appears to occur frequently in arthropods (Weddle et al. 2013). Based on how recognition takes place, self-referencing is used to identify kin vs. non-kin, but is not used in recognizing non-kin (Breed 2014). A second mechanism of recognition is through green beard effects. Coined by Dawkins, the term refers to a family-specific phenotype (e.g. a green beard) that allows individuals to recognize related individuals (Dawkins 1976). While out of favour for an extended period of time, it appears that green beard effects may occur in *Dictyostelium*, as matching alleles at a single specific locus are enough to induce cooperation (Strassmann et al. 2011). In this case, the level of actual relatedness may vary, but as long as individuals share their 'green beard' each will treat the other as related. In recognition by phenotype matching, individuals develop a template of what a 'relative' looks like and then compare conspecifics to that template to make decisions (Lacy & Sherman 1983). Interestingly, if the template is formed while in a group of non-relatives, phenotype matching may represent group recognition. Finally individual recognition implies that individual identities are learned and stored to memory, and that conspecifics can recognise specific individuals in subsequent encounters. Individual recognition is present in mammals, but has also been observed in vespid wasps, whose highly variable facial markings provide the variation necessary for learning individual identities (Tibbetts 2002; Sheehan & Tibbetts 2010).

The evolution of group formation

Prior to the evolution of reproductive skew and altruism, solitary organisms first come together and spend extended periods of time in groups. Three main theories have been proposed to facilitate social group formation: group augmentation, maternal manipulation and ecological constraint.

Group augmentation

The evolution of sociality by group augmentation suggests that the reproductive success of the individual increases along with group size (Kokko et al. 2001). Initial debate in the literature suggested that cheaters would quickly invade and erode this type of society, as genes for cheating would spread rapidly (Kokko et al. 2001). Other work has demonstrated that the evolution of social groups by group augmentation is possible when group size is small and the delay between helping and being helped is minimal (Kokko et al. 2001; Clutton-Brock 2009; Kingma et al. 2014). However, empirical tests of group augmentation in the literature are uncommon, and often coupled with benefits attained through indirect fitness benefits (Courchamp & Macdonald 2001; Browning et al. 2012).

Manipulation

The manipulation hypothesis proposes that colony members would like to disperse and reproduce on their own, but are in some manner forced to stay in the group as helpers. Generally, this manipulation takes the form of rendering the subordinate

individual unable to reproduce. If individuals are unable to reproduce upon dispersal, remaining at the natal site to help and gain at least some indirect fitness is the best option. Manipulation may take the form of physical abuse from an older or larger female (Clarke and Faulkes, 1997; Pabalan et al., 2000). For example, the constant 'nudging' performed by *Lasioglossum zephyrum* bee queens has been shown to render the ovaries of their workers inactive (Michener and Brothers, 1974). Fitness calculations from the sweat bee *L. malachurum* suggest that workers should lay eggs but are likely manipulated by the queen into remaining non-reproductive (Richards et al. 2005).

Ecological constraint

This hypothesis seeks to explain the formation of social groups through decreased dispersal and reduced survival in response to declining resources (Emlen 1982). It thus represents a type of bet hedging strategy whereby it is better to achieve reduced fitness through staying home and helping than attempting to disperse and failing to produce any offspring. Ecological constraint can be examined using many parameters. In the meerkat (Clutton-Brock et al., 1999) and dwarf mongoose (Rood, 1990), larger groups have been shown to increase colony survival when predator populations are high. Selection may also favour group living when nesting resources are scarce (McGlynn, 2010) or costly to establish (Gerling et al., 1989).

The evolution of reproductive skew and dominance hierarchies in social groups

Ecological and evolutionary forces can facilitate group formation, but how reproduction is partitioned among group members can have great consequences for

individual fitness. When reproductive opportunities are shared equally among group members, reproductive skew is low. These societies are often referred to as communal and have been reported from invertebrates (Abrams & Eickwort 1981; Kukuk & Sage 1994; Paxton et al. 1999) to vertebrates (Gilchrist 2006). Reproductive skew within the group increases as reproductive opportunities becomes disproportionally allocated to one or very few individuals. In highly eusocial insects such as honeybees, as well as many species of ants, reproduction is monopolized by a single individual. In some societies, such as in the wasp *Microstigmus nigropthalamus,* reproductive skew can even vary across nests (Lucas et al. 2011).

When reproductive opportunities are not shared equally among group members, competition for the largest piece of the reproductive pie can lead to the formation of dominance hierarchies, where the most dominant individual is also the most reproductive (Kokko & Johnstone 1999). This competition can lead to the formation of a linear queue behind the most dominant individual in the nest. If reproductive opportunities are correlated with queue position, being as close to the front of the queue as possible can increase potential reproductive output (Shreeves & Field 2002). In contrast, being at the back of a long queue can signal very limited opportunities for reproduction.

If the odds of reproduction are extremely low, natural selection can favour the evolution of other ways to attain reproductive opportunities. These can take a wide variety of forms, from individuals delaying reproduction (Woolfenden 1975) to evolving alternative phenotypes as illustrated by the sneaker male strategy seen in isopods, *Paracerceis sculpta* (Shuster 1989), bluegill sunfish, *Lepomis macrochirus* (Gross 1991) and the pygmy swordtail, *Xiphophorus nigrensis* (Ryan et al. 1992). This phenotypic and

behavioural variation can lead to the evolution of alternative or conditional reproductive strategies. An alternative strategy is defined as a heritable, genetically based system from which multiple phenotypes can be generated. For alternative reproductive strategies to persist, all must have equal average fitness (Gross 1996). Behavioural decisions made within conditional strategies are based on interactions with conspecifics and the environment and have no genetic predisposition. In conditional reproductive strategies decisions regarding which strategy to employ are based on the status of the individual at a given time and will improve the fitness of the individual in question. In this thesis I explore reproductive skew in eastern carpenter bees, and whether or not some members of the group employ a conditional reproductive strategy by delaying reproduction.

Kin selection as an explanation for the evolution of altruism

In established social groups with high levels of reproductive skew, many group members do not reproduce, meaning that they have no direct fitness opportunities (Michener 1974). Yet, in many social groups these individuals remain and often even help to raise offspring produced by others. This behaviour may seem contrary to the predictions of natural selection, where only strategies with the highest fitness prevail. The apparent altruism exhibited by non-reproductive helpers however can be explained by kin selection (Hamilton 1964a). Coined by Maynard Smith in 1964, the term kin selection predicts that an individual will act altruistically if it can pass on more genes identical by descent by helping to raise its siblings than by raising offspring independently. Hamilton (1964) further described the relationship in terms of the products of the cost and benefit of acting altruistically such that $r_k b > r_o c$, where r_k is the relatedness of the altruist to the

kin it helps to raise, b is the benefit to the individual the altruist is helping (usually calculated as the number of kin the altruist raises), r_o is the relatedness of the altruist to its own kin, and c is the cost to the altruist (the number of offspring it would have reared on its own). In haplodiploid species, singly mated females are more related to their sisters (r = 0.75) than to their own offspring (r = 0.5). However in diploid species we do not see this asymmetry between relatedness to offspring and sisters (r = 0.5 in both cases), which implies that the values of b and c can be critically important in order for kin selection to explain helping behaviour. While eusociality may be most commonly known from haplodiploid insects such as honey bees and ants it is also known from diploid species such as termites (Wilson 1971) and mole-rats (Jarvis 1981), implying that eusociality can evolve in a number of different conditions.

Since its arrival in 1964, the role of kin selection in the evolution of sociality has been a pervasive theme in sociobiology. Calculating all parameters (r, b and c) is difficult, and few studies have empirically tested Hamilton's rule. In the paper wasps, *Polistes dominulus*, the small probability of nest inheritance and direct fitness benefits explains why workers (even unrelated ones) remain at the nest to help (Leadbeater et al. 2011). For the wasp, *Ropalidia marginata*, helpers at the nest appear to be subfertile and actually obtain higher reproductive output when helping at the nest than when attempting to reproduce on their own (Gadagkar 2016). Yet in the small carpenter bee species, *Ceratina australensis*, it appears that females would have higher fitness by reproducing on their own than by staying to help (Rehan et al. 2014) unless under conditions of extreme parasitism (Rehan et al. 2011).

The role of competition in group composition

Kin selection theory suggests that cooperation should be higher among related individuals, but that competition among kin is a constant opposing force to kin cooperation (Hamilton 1964a; West et al. 2002). When resources are abundant and competition for them is low, kin selection predicts that cooperation among kin should be high. Comparatively, when resources are limiting, competition will outweigh the benefits of cooperation, resulting in increased levels of aggression among kin (West et al. 2001, 2002; Van Dyken 2010). This has been empirically demonstrated in male fig wasps, where increasing competition for mates leads to increased levels of aggression among brothers (West et al. 2001).

Increased levels of aggression can lead to injury of one or both interactants, which will in turn decrease individual fitness. One method of reducing competition among kin is through dispersal (Lena et al. 1998; Moore et al. 2006; Cote & Clobert 2010). By breaking up sibships, competition and therefore levels of aggression among kin are reduced. Examples of dispersal due to elevated levels of kin competition are rare, but have been reported for the fig wasp, *Platyscapa awekei* and the common lizard, *Lacerta vivipara*. In *L. vivipara*, individuals dispersed when kin competition was high even when dispersal risk was elevated, demonstrating the influence of kin competition on behavioural decisions (Cote & Clobert 2010). In this thesis I investigate the factors influencing social group formation in *X. virginica*, incorporating the concept that competition among kin may influence the final composition of social groups.

Model systems to study the evolution of sociality

Many different kinds of social groups can be found in nature. These vary from reproductive aggregations, which are essentially solitary individuals reproducing near one another, to highly eusocial organisms where a single female monopolizes all reproduction (complete reproductive skew) and is surrounded by irreversibly sterile workers which help her to rear her young (Michener, 1974). A few species have maintained the ability to display flexible life histories. Socially polymorphic species are able to reproduce both solitarily and in social groups and are useful species to investigate transitions to social living, as the costs and benefits of different strategies can be tested within a single species. Asking questions relating to social transitions using organisms that are obligately social may lead to erroneous results, as selection pressures may have changed since traits became fixed (Linksvayer and Wade, 2005; Michener, 2007). This thesis uses the facultatively social eastern carpenter bee, *Xylocopa virginica,* as a model to investigate the evolution of sociality.

***Natural history and colony cycle of the eastern carpenter bee,* Xylocopa virginica**

Xylocopa virginica is a large generalist pollinator whose range encompasses most of eastern North America (Michener 2007). Members of the Apid subfamily Xylocopinae, the genus is the only member of the tribe Xylocopini, which is basal to the other three tribes within the Xylocopinae (Manueliini, Ceratinini and Allodapini; Rehan et al 2012). Primarily a tropical genus, *Xylocopa virginica* is one of five species of *Xylocopa* present in Canada and the United States, and one of only two species found in eastern North America (Michener 2007). Eastern carpenter bees excavate nests in wood

and while historically preferring to nest in pine and cedar (Hurd 1961), they now nest almost exclusively in milled lumber, linking them with anthropogenic disturbance across their range.

In the northern portion of their range, eastern carpenter bees have one reproductive period per year, and bees overwinter as adults inside the nest. In spring (typically mid to late April) bees become active and are seen outside the nest. The first three to four weeks is termed the nestmate provisioning phase, when females bring pollen back to the nest to feed to other adults rather than for provisioning offspring (Richards & Course 2015). During this time most of the dispersal takes place, with many bees leaving the population entirely, or joining new nests within the aggregation (Peso & Richards 2010b). Late May until late June or early July is the brood provisioning phase, when pollen brought back to the nest is used for provisioning offspring. *Xylocopa virginica* are mass provisioning bees, meaning that all resources required to develop from egg to adult are supplied by a large pollen ball when the egg is laid (Rau 1933; Gerling & Hermann 1978). Once offspring provisioning is complete, females remain inside the nest to guard the developing juveniles. Offspring emerge as adults from late July through August and typically overwinter in their natal nest.

Three different types of females have been described in social nests, which may represent two different reproductive strategies (Richards 2011; Richards & Course 2015). Primary females are both the dominant forager and egg layer in the nest and have the first opportunity at direct fitness benefits. Secondary females appear to queue for reproductive opportunities behind dominant females, and are seen flying outside the nest and occasionally making pollen trips (Richards 2011; Richards & Course 2015). Tertiary

females do not forage or leave the nest, but can successfully overwinter twice, essentially doubling their lifespan.

Perhaps most interesting about eastern carpenter bees is their ability to display both solitary and social phenotypes within the same nesting aggregation. This behavioural plasticity makes them an excellent species with which to investigate the evolutionary and ecological pressures underlying the formation of social groups and reproductive skew, as females can choose to nest socially or solitarily. Describing the factors that lead to solitary versus social nesting in *Xylocopa virginica* will provide new details as to how sociality evolves within a species, a valuable contribution to the field of social evolution.

Thesis objectives

This thesis has two main objectives. My first main objective is to describe the population genetic structure of *X. virginica* across its range and to investigate whether climate characteristics can describe the population genetic structure seen among populations. This objective is investigated in chapter two, where I use species-specific microsatellite markers developed during my PhD (Appendix) to describe the genetic differentiation of carpenter bees across their range.

The second main objective of my thesis is to investigate proximate and ultimate factors that contribute to the evolution and maintenance of social groups in *Xylocopa virginica*. Chapters three to five each explore a different component of social nesting in eastern carpenter bees. Chapter three aims to examine how females recognize one another in social nests, specifically whether they use kin or non-kin based cues. How

females recognize one another in the nest can affect which bees are accepted into new nests during spring dispersal, as well as group stability across the season.

Behavioural interactions among conspecifics also influence the dominance status of females in social nests, leading to differences in reproductive opportunities among females. In *X. virginica,* it may to have led to the evolution of two different reproductive strategies. Chapter four aims to describe the reproductive queue and to understand how females respond when new reproductive opportunities arise within the nest. This chapter will provide novel information about the flexibility of reproductive strategies within the nest and the physical characteristics of the females employing them.

Finally, chapter five aims to understand the ultimate ecological and evolutionary drivers of sociality in eastern carpenter bees. Typically, kin selection is used to explain the evolution of cooperation in social groups, but the role of kin competition during the evolution of sociality is less explored. For eastern carpenter bees costly nesting resources and short breeding seasons may lead to high levels of competition among kin, which may in turn influence the final composition of social groups. I hypothesize that competition among kin plays an important role in shaping the evolution of social groups in this species.

Rationale for chapter two

In light of the decline of many native pollinators, there is a push to understand the population genetic structure of native bees (Potts et al. 2010). To date, most of this research has focussed on species known to be in decline, or specialist species that may be at particular risk of habitat fragmentation (Packer & Owen 2001; Packer et al. 2005; Exeler et al. 2010; Dellicour et al. 2015a). These studies have all examined how human changes to natural habitat have affected population structure, but little is known about the population structure of species that are associated with human disturbance. *Xylocopa virginica* now nests almost exclusively in milled lumber, a substrate that is only available due to anthropogenic disturbance. Generalist pollinators with a large geographic range, eastern carpenter bees are common throughout eastern North America. The link between human disturbance and *X. virginica* nesting substrate offers a unique opportunity to understand the population structure of a native pollinator that is associated with anthropogenic disturbance.

Chapter 2: Nesting habits influence population genetic structure of a bee living in anthropogenic disturbance

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Author contributions: JLV and MHR designed the experiment. JLV collected and genotyped specimens and analyzed the data. MHR provided equipment and reagents. JLV and wrote and MHR edited the manuscript.

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Introduction

Anthropogenic disturbance continually modifies how genes flow through landscapes, typically leading to decreases in genetic diversity across populations (Greenwald *et al.* 2009; Winfree *et al.* 2009; Spear & Storfer 2010). Disturbance can reduce the amount of suitable habitat available to a species, alter habitat connectivity, or both. Habitat fragmentation often produces smaller, genetically isolated populations with lower genetic diversity and increased levels of inbreeding (Dixon *et al.* 2007; Dixo *et al.* 2009; Haag *et al.* 2010), while contracting geographic ranges can support fewer individuals, thus reducing effective population size. Factors affecting population structure can alter gene flow at different scales, within local populations, among populations within a given region, or across the entire distribution of the species.

Despite their importance to both natural and agricultural systems, populations of wild pollinators are in widespread decline, with anthropogenic impacts being implicated as the primary driving force (Whitehorn *et al.* 2009; Davis *et al.* 2010; Kennedy *et al.* 2013; Rader *et al.* 2013; Goulson *et al.* 2015; Kerr *et al.* 2015; Park *et al.* 2015; Koh *et al.* 2016). For most wild bees, the major cause of population declines is habitat loss which results in smaller demographic and effective population sizes (Ne) and loss of genetic diversity (Packer & Owen 2001; Zayed *et al.* 2005). Another problem is habitat fragmentation, which can reduce genetic connectivity between populations and can lead to greater population genetic differentiation among populations (Fischer & Lindenmayer 2007). For bees, the negative population genetic effects of habitat loss are likely magnified by the fact that, as a group, they have low genetic diversity, even in comparison to other hymenopteran insects, which have relatively low genetic diversity

due to their haplodiploid genetic systems (Packer & Owen 2001; Zayed *et al.* 2004; Zayed & Packer 2005).

Although recent research has mostly uncovered negative demographic and population genetic effects, the effects of anthropogenic environmental change on bees are not universally negative - some species persist or even thrive in human-altered habitats. "Anthrophilic" species often exhibit specific biological characteristics that help them thrive in human-dominated landscapes, such as the ability to capitalize on food or shelter provided by humans (Gardner-Santana *et al.* 2009; Rochlin *et al.* 2013). Recently, studies have demonstrated relatively high levels of bee diversity in cities (Matteson & Ascher 2008; MacIvor & Packer 2015), while habitat restoration projects have provided ample evidence that bees rapidly colonize newly available habitat even in urban and suburban areas (Richards *et al.* 2011; Geroff *et al.* 2014). This suggests that bee populations are not simply sensitive to habitat loss, but more generally, are responsive to the availability of critical resources such as food and nest sites. Human activity usually decreases resource availability, but sometimes the opposite occurs, and human activity increases habitat and resource availability. The population consequences of increased habitat or increased availability of critical resources may include demographic increase, range expansion, migration, and establishment of new populations, all of which may carry detectable population genetic signatures.

Links between bee population structure and the spatial distribution of critical resources has so far been studied only in foraging specialists (Stow et al. 2007, Davis et al. 2010). Specialist foragers are predicted to be patchily distributed in space, because all else being equal, their food resources should be distributed more patchily than the

food resources of generalist foragers. As a result, specialist bee populations should have lower population sizes and should exhibit higher levels of population differentiation (F_{ST}) between populations than generalist bees (Packer & Owen 2001; Packer *et al.* 2005). However, there is increasing evidence that specialists do not necessarily live in fragmentary populations, as some exhibit much lower levels of population differentiation than expected (Exeler *et al.* 2008, 2010; Černá *et al.* 2013; Dellicour *et al.* 2014). Thus population structure likely depends not on foraging specialization per se, but on the spatial distribution of specialized food resources. Two recent examples illustrate how human-generated increases in floral resources have influenced their bee specialists. The geographic range of the ivy foraging specialist *Colletes hederae* is closely tied to that of its exclusive host plant *Helix hedera*, which is very widespread and abundant because of its popularity with gardeners (Dellicour *et al.* 2014). The abundance and distribution of the host plant has facilitated a recent rapid expansion in both the range and population size of *C. hederae*, with little evidence of genetic differentiation among populations or loss of genetic diversity in recently founded populations (Dellicour *et al.* 2014). A similar story is found in the squash specialist bee *Peponapis pruinosa,* where range expansion by its host plant for agriculture facilitated rapid range expansion of the bee as well (López-Uribe *et al.* 2016).

The role that nest site specialization plays in shaping population genetic structure has not previously been explored. Specialized nesting resources, like specialized foraging resources, have spatial distributions that may affect bee population genetic structure, especially if they are patchily distributed or are rare. Most bees dig burrows in the ground and exhibit no obvious preference for specific site characteristics, but others

exhibit very specific microhabitat preferences, choosing particular soil types, constructing burrows in specific types of wood, or searching for pre-existing cavities of specific sizes (Potts & Willmer 1997; Michener 2007). Additionally, both solitary and social bee species often nest in aggregations (Michener 2007). As many bees are philopatric, nest aggregations may persist for many generations; this can lead to genetic differentiation among aggregations and among populations in different locations, or to isolation by distance, depending on how long nesting aggregations usually persist and on rates of migration among them (Stow *et al.* 2007; Zayed & Packer 2007; Davis *et al.* 2010). Moreover, population sizes and even geographic range are likely to be constrained by the availability of a scarce or extremely patchy nesting resource. If such a nesting resource were suddenly made more abundant by human or other influence, then the populations of bees dependent on that resource would be expected to grow, which in turn, could influence population genetic structure.

Objectives

In the current study, we investigated the population genetic structure of the eastern carpenter bee, *Xylocopa virginica,* which thrives in association with humans. *Xylocopa virginica* is a large species whose range covers much of the eastern United States as far north as southern Ontario, Canada. It is a foraging generalist, but a nesting substrate specialist, and it now nests almost exclusively in structures built from milled lumber, especially pine and spruce (Hurd 1978). As a result, the population distribution of *X. virginica* is now strongly linked to anthropogenic modifications of habitat. Carpenter bees are strongly philopatric, nests are occupied by successive generations and

new nests are often constructed in close proximity to established ones, creating nesting aggregations that may persist for years and even decades (Rau 1933; Richards & Course 2015). Large nesting aggregations tend to be found in wooden structures such as old barns, wooden bridges, and park benches (Richards 2011; Skandalis *et al.* 2011), which are not evenly distributed across landscapes. To investigate population genetic structure across the geographic range of *X. virginica*, we used nine species-specific microsatellite markers to look for genetic variation within and among 16 different populations, from Ontario at the northern edge of the range, south to Florida at the southern edge and west to Arkansas. We predicted that philopatric nesting, together with the patchy distribution of wooden structures that can support large nesting populations, should result in significant genetic differentiation among populations and could also lead to isolation by distance. Given previous evidence that variation in temperature and precipitation helps to explain *X. virginica*'s geographic range (Skandalis *et al.* 2011), we investigated whether these climatic variables were useful in explaining range-wide genetic structure observed in carpenter bees. We found evidence for significant genetic differentiation among populations and by distance, suggesting that nesting habits do indeed have a strong influence on population structure. We also found that carpenter bee populations cluster into three distinct geographical regions, possibly implying range expansion to the north and west.

Methods

Specimen collection

Adult female *Xylocopa virginica* were collected from 16 different populations from across south-eastern North America between April and June in 2011- 2013 (Table 2.1). Collections took place on sunny days when the air temperature was higher than 20^{o}C. Bees were caught on the wing while foraging and immediately placed in redistilled 99% ethanol. We aimed to collect at least 20 females per population, but fewer specimens were collected where local population sizes were small. Collection sites were typically located in gardens where many females were seen foraging on flowers, and so collections at a particular site may represent multiple nesting aggregations.

Xylocopa virginica is facultatively social and typically there is only one forager per nest at any given time (Richards & Course 2015), so the probability of collecting nestmates was low. Moreover, colonies of *X. virginica* are composed mainly of non-relatives (J Vickruck and MH Richards, unpub. data), further lowering the chances of collecting related individuals. Colony 2.0.3.4 was also used to detect the presence of full sisters in the dataset using the very high precision method and assuming random mating (Jones & Wang 2010). Of the 328 females collected, Colony detected 7 pairs of potential full siblings in the dataset. Ten sample populations contained no evidence of full siblings in the dataset, five populations showed evidence for one set of siblings and one population showed evidence for two pairs of siblings. To test the effect that these seven sibling pairs may have on the dataset, one female from each pair was removed and pairwise population differentiation was calculated by F_{ST}. Removal of the seven females

did not change the significance pattern of population differentiation seen among populations, and changed the overall mean F_{ST} so slightly that it was not detectable after rounding. As such all females were retained for downstream analysis.

DNA extraction and amplification

DNA was extracted from a single metathoracic leg with the Qiagen DNeasy blood and tissue kit using the manufacturer's recommended protocol with the addition of the following step: after tissue lysis with buffer and proteinase K, the supernatant was transferred to a clean microcentrifuge tube to prevent bits of exoskeleton from clogging the spin column in subsequent steps.

Genomic DNA was amplified at nine microsatellite loci previously described by Vickruck (2015). Each locus was amplified in a single 15µl PCR reaction using 40-70 ng of DNA, 1 unit standard Taq (New England Biolabs), 1x Thermo Buffer (New England BioLabs), 0.2 mM dNTPs, 0.2 µM forward primer, and 0.2 µM reverse primer. Forward primers contained a fluorophore (either 56-FAM or HEX) to make them detectable during electrophoresis. PCR conditions were as follows: 95°C for 3 min, then 40 cycles of 94°C for 30 sec, 52 or 55°C for 30 sec, and 72°C for 30 sec. PCR reactions for loci XV23, XV39, XV42, XV43, and XV3 used annealing temperatures of 52°C, while those for loci XV24, XV27, XV7, and XV30 used annealing temperatures of 55°C. Labelled PCR products were run on an Applied Biosystems 3730xl DNA Analyzer at the Peter Gilgan Centre for Research and Learning Genetic Analysis Facility. Each PCR reaction contained two positive controls, individuals that had already been genotyped at that locus, enabling detection of shifts among runs. Alleles were called using GeneMapper v3.5 and

then checked by eye. Twenty-eight individuals were re-genotyped at all loci to confirm accuracy of allele calls. Genotypes produced for those 28 individuals at all 9 loci were identical to those from the first genotyping run.

Allele and locus characteristics

Tests for Hardy-Weinberg equilibrium and linkage disequilibrium at each locus pair in each population were performed using Genepop v4.1 (Raymond & Rousset 1995). Observed and expected heterozygosities within each population and locus were calculated in GenAlEx v6.5 (Peakall & Smouse 2012). GenAlEx was also used to detect private alleles at the population level and to calculate F_{IS}. HP-Rare was used to calculate rarefied allelic richness, accounting for differing sample sizes in our sample populations (Kalinowski 2005).

Effective population size was estimated using the sibship assignment method assuming random mating using COLONY 2.0.3.4 (Jones & Wang 2010). This method assumes that individuals are taken from a single cohort at random. Two populations (OH and GA) returned values of infinity, which is most likely due to small sample sizes of these populations (Jones & Wang 2010).

Genetic isolation and bottlenecks

We tested for isolation by distance (IBD) by investigating if there was a relationship between pairwise genetic distance and pairwise geographic distance among our sample populations. We first created a matrix of pairwise Euclidian genetic distances among populations using the *dist.genpop* function of the R package adegenet v1.4-2

(Jombart & Ahmed 2011). We then used a Mantel test to compare our matrix of genetic distances to a matrix of pairwise geographic distances using the *mantel.randtest* function in the R package ade4 v1.6-2 (Dray & Dufour 2007).

We used the program Bottleneck to look for evidence of recent genetic bottlenecks within populations (Piry *et al.* 1999). This method looks for deviations from expected heterozygosities as generated under mutation-drift equilibrium (HetEQ) based on both sample size and the number of alleles at each locus. Calculated HetEQ values are averaged over loci and then compared to the observed heterozygosity. We used the Two-Phase Model, which is most realistic for mutational events in microsatellite loci (Piry *et al.* 1999). The two phases were set to 95% single-step mutations and 5% multiple-step mutations and run for 10 000 iterations for each population sampled. Significance was assessed using Wilcoxon test results, which are most appropriate when the number of loci in the dataset are under 20 (Piry *et al.* 1999).

Genetic differentiation among populations and across the geographic range

Several methods were used to investigate genetic differentiation. Pairwise differentiation among sample populations was measured using F_{ST} and G'_{ST} in GenAlEx v6.5 (Peakall & Smouse 2012). For highly polymorphic loci, the maximum value of F_{ST} depends on the number of alleles per locus. Therefore, we also calculated D_{EST} to standardize among loci with differing numbers of alleles (Jost 2008; Heller & Siegismund 2009). Significance of pairwise $F_{ST,}$ G'_{ST} and D_{EST} comparisons was assessed using 9999 permutations.

To estimate the number of genetic groups (*K*) represented by all sampled populations, we used a Bayesian clustering method implemented in Structure 2.3.4 (Pritchard *et al.* 2000). Ten runs of *K*=1 through *K*=16 (160 total runs) were performed with a burn-in of 50 000 iterations followed by 1 000 000 Markov Chain Monte Carlo iterations under a model of correlated allele frequencies and admixture. Using the output from the Structure runs, the true number of populations (*K*) was inferred using the ln *K* (Pritchard et al. 2000) and Evanno (2005) methods. The ln K method infers *K* based on the highest mean log-likelihood, while the Evanno method uses the rate of change of the log posterior probability across increasing *K* values. Graphical representations of population membership generated by Structure were created using Clumpak (Kopelman *et al.* 2015), which can account for node switching and create average plots for each of the 10 trials run for each of the hypothetical 16 values for *K* (Kopelman *et al.* 2015). Initial runs indicated that the Ontario (ON) population was very different from the remaining populations. One of the identified weaknesses of the Structure algorithm is that it can have difficulty detecting hierarchical population structure (Evanno *et al.* 2005). To ensure that strong genetic differentiation of the Ontario population was not masking additional structure in the dataset, Ontario bees were removed and a second set of Structure analyses were conducted with the same parameters on the reduced dataset.

In addition to Structure, we employed Geneland to infer population structure (Guillot *et al.* 2005). Geneland is similar to Structure in that it uses Bayesian clustering techniques, but it incorporates spatial coordinates of each population into the model. Each Geneland simulation returns the most probable number of genetic groups given the data. We used both the correlated and uncorrelated allele models with the range for

possible populations (*K*), set from 1 to 16 (the number of populations sampled); the correlated allele model in Geneland can detect more subtle population differentiation when compared to other Bayesian clustering programs, but is more sensitive to isolation by distance (Latch *et al.* 2006). Uncertainty of geographic coordinates was set to 0 as all individuals from a population were collected from the same site. The null model was set to true. The final model was based on 1 000 000 Markov Chain Monte Carlo iterations and a thinning value of 100. These parameters were replicated in five separate simulations to compare the similarity of results across runs.

To look at the partitioning of variance among the sampled populations we used Analysis of Molecular Variance (AMOVA) in Arlequin 3.5.12 (Excoffier & Lischer 2010), comparing genetic variation within and among the geographic groups inferred from Structure and Geneland.

Lastly, we used GESTE v2.0 (Foll & Gaggiotti 2006) to investigate the influence of spatial factors on genetic differentiation in eastern carpenter bees. GESTE v2.0 estimates population specific F_{ST} values using hierarchical Bayesian methods and a generalized linear model to generate posterior probabilities for all combinations of the given variables (Foll & Gaggiotti 2006). Significant differentiation across latitude or longitude has been interpreted as evidence for range expansion (Foll & Gaggiotti 2006). Here, GESTE v2.0 was used to compare models that included latitude only, longitude only, both latitude and longitude and the interaction between the two. The final model included the reversible jump and 10 pilot runs with a burn-in of 50 000 iterations, sample size of 10 000 and a thinning interval of 20.

Climatic variables as explanatory factors of population structure

We used distance-based redundancy analysis (dbRDA) implemented with the function *capscale* in the R package vegan v2.2-1 to investigate how much of the genetic differentiation among populations was explained by environmental variables (Oksanen *et al.* 2012). Classically a tool used in community ecology, redundancy analysis has been adopted as a powerful tool for landscape genetic analyses (Kierepka & Latch 2015). dbRDA combines regression and principal components analysis to allow genetic variance among populations to be examined in a model with multiple explanatory variables. The model comprised the pairwise genetic distance matrix as the response variable, with mean monthly temperature (^{o}C) for both summer (April-October) and winter (November-March), as well as total monthly precipitation (mm) for both summer and winter as explanatory variables. Significance was tested using 999 permutations with the function *anova.cca* in the R package ade4 v1.6-2 (Dray & Dufour 2007). Climate data for the years 1974-2014 for weather stations as close to the collection sites as possible were acquired from the National Oceanic and Atmospheric Association database (ncdc.noaa.gov) (Supplementary Table S2.1).

Results

Allele and locus characteristics

Altogether, 133 different alleles were scored across nine loci (mean 14.8 ± 3.6 alleles/locus, range 10–21; Table 2.2). Private alleles were detected in 11 of the 15 populations sampled, with the Kentucky (KY) population containing four, the highest number (Table 2.1). Observed and expected heterozygosities by population ranged from

0.488–0.785 and 0.552–0.791, respectively (Table 2.1). Inbreeding, as measured by F_{IS} within each population, was low (mean 0.010±0.06, range -0.124–0.116; Table 2.1). Estimated effective population sizes (Ne) ranged from 18 in Ontario (ON) to 285 in Maryland (MD; Table 2.1).

Tests for departure from Hardy-Weinberg equilibrium (HWE) at each locus in each population revealed 26 significant results among the 144 tests conducted. After Bonferroni correction, only three population-locus comparisons remained significantly different from the expectations of HWE. No locus was consistently out of HWE across all populations. Significant linkage disequilibrium was detected in 21 of the 576 population-by-locus tests. There was no evidence that particular pairs of loci or populations showed linkage disequilibrium. Therefore, all data were retained in further analyses.

Population differentiation

Multiple analytical methods generated similar patterns of genetic differentiation. Pairwise population-level F_{ST} values ranged from 0.013–0.16 (mean F_{ST}=0.11, Supplementary Table S2.2). In particular, the Ontario (ON), Iowa (IA), and Arkansas (AR) populations were significantly differentiated from all other populations. The Georgia (GA) population was significantly differentiated only from the Ontario, Iowa and Arkansas populations (Supplementary Table S2.2), probably due to the small number of bees in this sample (n=6). D_{EST} comparisons showed pairwise values that ranged from 0.008–0.596 (population level D_{EST}=0.17; Table S2.2), and the pattern of pairwise

significance among populations was exactly the same as when calculated via F_{ST} (Supplementary Table S2.2).

Structure analyses indicated that the 16 sampled populations comprised *K*=3 or *K*=4 groups, as inferred by the Evanno (2005) and Pritchard *et al.* (2000) methods respectively (Figure 2.1). The map in Figure 2 shows the geographic pattern of group membership. When *K*=3, the Ontario (ON) population formed the Northern group on its own, the Iowa (IA) and Arkansas (AR) populations formed the Western group, and the remaining populations formed one Core group. When *K*=4, the Core group was subdivided into two subgroups, comprising an Eastern Core group containing the NC(R), NC(G), SC(G), VA, PA, GA, FL, and MD populations, and a Western Core group containing the SC(S), NC(K), KY, TN, and OH populations. With either analytical method, omitting ON, the most genetically distinct population, reduced *K* by 1 but did not change the membership of the Western or Core groups.

Additional analyses with Geneland supported the outcomes of the Structure analyses (Supplementary Figure S2.1). Analyses based on the correlated allele model suggested that K=16 (each sample populations represents a separate group). Using the more relaxed uncorrelated allele model, all simulations indicated that K=3 (mean log posterior probability -8893.7±4.3, n=5 runs). Group membership was the same as that produced by Structure when *K*=3.

GESTE v2.0 suggested that the model including the constant and latitude had the highest posterior probability of all models tested (Table S2.3). Latitude also had the highest posterior probability of the individual factors tested (Table S2.3).

Based on the findings from Structure and Geneland, we used hierarchical Analysis of Molecular Variance (AMOVA) to investigate how genetic variance was partitioned in and among groups and populations. With *K*=3 groups (Northern, Western, and Core) or *K*=4 groups (Northern, Western, Eastern Core, and Western Core), all variance components were significant in both analyses, although the percent of variation among groups was substantially lower with four than with three groups (Table 2.3). When we compared just the Eastern and Western Core groups, there was no significant variation between groups (Table 2.3c).

Isolation by distance and lack of evidence for recent bottlenecks

Isolation by distance was revealed by a correlation between genetic and geographic distances based on all pairs of the 16 populations (Mantel test, r = 0.53, simulated P=0.005; Figure 2.3). Significant isolation by distance was also detected when limited to just the populations belonging to the Core group (Mantel test, r = 0.29, simulated P=0.04; Figure 2.3).

The program Bottleneck detected no genetic bottleneck in the last ~40 generations in any of the 16 populations (Table S2.4).

Temperature and precipitation as explanatory factors for population genetic structure

Distance-based redundancy analysis (dbRDA) based on a model including all four climatic variables (mean monthly summer and winter temperatures as well as mean monthly summer and winter precipitation), was not statistically significant (Pseudo $F_{(4,11)}$=2.12, P=0.15). Since Skandalis *et al.* (2011) previously showed that temperature is

a much stronger predictor of population distribution, we ran a second analysis that included only the terms for mean monthly summer and winter temperatures. This model was significant (Pseudo $F_{(2,13)}$=3.74, P=0.046), with variation in temperature explaining 27% (R^2_{adj}) of the genetic variation among populations (Figure 4). The distribution of population vectors across the first two axes of the redundancy analysis mirrored the three genetic groups indicated by previous analyses; ON loaded positively on axis 1 and IA and AR loaded positively on axis two (Figure 2.4).

Discussion

The relationship between nesting habits and population genetic structure

The philopatric nature of *Xylocopa virginica* nesting behaviour, coupled with the patchy distribution of structures built of milled lumber, led us to predict that eastern carpenter bee populations should be genetically differentiated from one another and show isolation by distance. These predictions were supported. As expected, most inter-population comparisons in our data set showed significant differentiation even over short distances, which we attribute to nesting substrate specialization combined with philopatry. While foraging specialization is commonly predicted to induce population genetic differentiation in bee populations (Zayed *et al.* 2005; Dellicour *et al.* 2015), nesting biology is also an important determinant of population structure. Much of the previous research on inter-population structure of bee populations has focused on foraging specialist or species of special conservation concern (Danforth *et al.* 2003; Zayed *et al.* 2005; Zayed & Packer 2007; Exeler *et al.* 2010; Lozier *et al.* 2011; Suni & Brosi 2011; Černá *et al.* 2013; Dellicour *et al.* 2014; López-Uribe *et al.* 2016). This is

the first study to examine the influence of nesting resource specialization on population structure.

Although estimated effective population sizes of *X. virginica* varied among sample populations, most were small (Ne<100 in 13/16 populations). Small effective population sizes likely result from both the local availability of nesting substrate and from the bee's nesting behaviour. The actual physical size of potential nest sites such as old barns, gazebos, sheds, and benches, limits the numbers of nests in aggregations. Moreover, most *X. virginica* colonies contain multiple adult females, but only one female at a time forages and lays eggs (Gerling & Hermann 1978; Richards & Course 2015), and many females likely never reproduce at all (Richards and Course 2015). Similarly, males are territorial, and competition for mates restricts the number of individuals that actually breed (Barrows 1983). Thus both maternity and paternity are restricted within populations, which could explain why effective populations are usually low.

Given that Ne tended to be low, it was somewhat surprising that in most populations there was no evidence for inbreeding. Male carpenter bees are territorial and often guard areas near their natal nests, where they spent the previous night (Peso & Richards 2010) which may increase the chances of brother-sister mating in aggregations. However, males also move from nest to nest over the course of the mating period, which could help reduce inbreeding in the long run (Peso & Richards 2010). The population with the smallest effective population size (Ontario) exhibited the highest level of inbreeding in the dataset, perhaps because in small populations, avoidance of siblings during the mating season is more difficult.

Population genetic differentiation within the Core group

Tests of genetic structure across the geographic range showed three regional genetic groups: a Northern group comprising the Ontario population, a Western group comprising the Iowa and Arkansas populations, and a Core group of the remaining 13 populations. The Core group was the largest group of populations identified and represents what is likely the ancestral home range of *X. virginica*. Georgia was the least differentiated population, which probably reflects its small sample size. Within the Core group there was significant isolation by distance. One possible explanation for this is a trade-off between philopatry, which would limit gene flow among populations, and dispersal, which would increase gene flow among populations.

Within the Core group, somewhat equivocal evidence emerged that the Appalachian mountain range, the most prominent major land form in eastern North America, may serve as a barrier to gene flow between eastern and western populations of *X. virginica.* Structure analyses based on the ln *K* method (Pritchard *et al.* 2000) suggested that the Core group might be further subdivided roughly east and west of the Appalachian mountain range. The initial AMOVA also supported this division, although the differentiation between East and West Core groups disappeared when the dataset was pared down to Core groups only. However, two populations assigned to the Western Core group (NC(K) and SC(S)) are actually located east of the Appalachians, so if the mountains do represent a barrier to gene flow, it is not a perfect one. Soltis et al. (2006) conducted a meta-analysis to examine patterns of population differentation for many species across the Appalachian mountain range. They found that while several patterns of population differentiation emerged, no pattern best described all species. The east-west

differentiation pattern is likely due to there being two distinct refugia on the east and west sides of the Appalachian mountain range, or two separate migration routes, one east and one west of the Appalachian mountains, that were used during northward re-colonization after the last glaciation. Further fine-scale sampling along the east-west axis of the Appalachian mountain range would be useful to refute or support this scenario. The fact that two eastern carpenter bee populations are more closely associated with the Western Core group may represent unintentional relocation by humans. There are now multiple examples in which bees that nest in lumber have been transported great distances, resulting in the establishment of new populations (Gibbs & Sheffield 2009; Sheffield *et al.* 2010), which would lead to discordant patterns of population strucure.

As a comparison, the bumblebees *Bombus bimaculatus* and *B. impatiens* have geographic distributions similar to that of *X. virginica*, but neither bumblebee species shows any discernible genetic structure across its range (Lozier *et al.* 2011). Although bumblebees are superficially similar to eastern carpenter bees in size, colouration, and foraging habits, their biology is quite different: they nest primarily in pre-existing cavities in the ground (Williams *et al.* 2014) and do not reuse their nests or nest in aggregations. Since they are not philopatric, young bumblebee queens are more likely to disperse away from their natal nests than female carpenter bees, which in itself should lead to more population mixing, at least at local levels. Moreover, the nesting resources of bumblebees are probably relatively evenly spread across landscapes, which would tend to mitigate population fragmentation. All in all, the major differences in life habits of carpenter bees and bumblebees likely explain why their populations show very different patterns of geographic structure.

Evidence for range expansion

The finding that three edge populations (ON, IA, and AR) belonged to groups genetically distinct from the Core group suggests that eastern carpenter bees may be undergoing range expansions northward and potentially westward. Range expansions are often detectable via a distinct genetic signature. First, peripheral populations typically show high levels of differentiation from central populations owing to genetic drift and mutation surfing at the expansion front (Excoffier *et al.* 2009; Hallatschek & Nelson 2010). Second, populations at the expansion front often exhibit reduced allelic diversity (Austerlitz *et al.* 1997; Excoffier *et al.* 2009). Lastly, recently expanded populations may show signs of inbreeding and bottlenecks if they are fragmented from the core population and have reduced opportunity for genetic exchange (Austerlitz *et al.* 1997).

The Northern group showed the strongest evidence for a range expansion in our analyses. Both distribution-wide assignment tests (Structure and Geneland) indicated that the Northern population is distinct from the Western and Core groups. Pairwise F_{ST} values between Ontario and all other populations were among the highest in the dataset. The Northern population had the lowest allelic diversity of any sample population, along with the highest levels of inbreeding. Bayesian generalized linear models (GESTE v2.0) also revealed that latitude significantly predicted population genetic structure. The only result which was not congruent with an inference of northward range expansion was the inability to detect a genetic bottleneck. However, the window for detecting recent bottlenecks using differences in observed and expected heterozygosities in mutation-drift equilibrium is typically only a few dozen generations (Luikart *et al.* 1998; Piry *et al.*

1999). Coupled with the short generation time of *X. virginica* (most individuals breed at 1 year of age), this means that only bottlenecks that occurred in the last 40 years are likely be detected. Historical records of *X. virginica* in southern Ontario date back to 1905 (Skandalis *et al.* 2011), so it is possible that if a bottleneck occured during the initial colonization over 100 years ago, it would now be undetectable. However, the Northern population (ON) also had the smallest effective population size in the dataset. Species with faster growth rates and large numbers of migrants more quickly overcome the genetic consequences of a range expansion than those with slower growth rates and small numbers of migrants (Nei *et al.* 1975; Austerlitz *et al.* 1997; Excoffier *et al.* 2009). Smaller sized populations at the front of a range expansion also tend to have lower levels of genetic diversity (Hallatschek & Nelson 2008), as seen in the Ontario population.

The analyses of climatic data indicated that a significant amount of the genetic differentiation among sample populations was associated with variation in both summer and winter temperatures. Winter temperatures certainly impose a northern boundary to the geographic range, as carpenter bee populations cannot persist where winter minimum temperatures fall below about -29^{o}C, the winter supercooling point of *X. virginica* (Skandalis et al. 2011). Subtle biological differences have been noted in supercooling points for carpenter bees collected in Ontario versus those collected in Maryland (Skandalis *et al.* 2011). Although neutral microsatellite loci do not tell us about selection, genetic differentiation at the periphery of the population, coupled with environmental differences across the range, suggest that local adaptation may be playing a role in some of the genetic differentiation among carpenter bee populations. In particular, the three Northern and Western populations at the periphery of the geographic range are

genetically distinct and experience climatic conditions that differ the most from the Core populations. Additional research into the morphological, behavioural and physiological characteristics of peripheral populations is needed to further understand whether local adaptation may be taking place in this species.

Breeding season length, which is a function of both winter and summer temperatures (or more precisely, spring and autumn temperatures) may be another important climatic influence which would have its strongest effect on northern carpenter bee populations (Skandalis et al. 2011). Historical records (Skandalis *et al.* 2011), as well as behavioural data (Gerling & Hermann 1978; Richards & Course 2015), suggest that in northern populations emergence from hibernation occurs as much as two months later than in southern populations. Eastern carpenter bees have relatively short activity seasons (approximately 8-10 weeks in southern Ontario), but brood have long developmental times (~45 days; J Vickruck unpub., data) and must eclose as adults early enough that they can feed before the onset of hibernation. In Ontario, young carpenter bees may reach adulthood only a few weeks before early frosts, so the shortness of the breeding season limits northward expansion. On the other hand, climate change, which has already resulted in perceptibly earlier springs and longer summers in Ontario (Richards *et al.* 2015), could be promoting demographic expansion of populations at the northern edge of the range. Certainly, anecdotal evidence suggests that in Ontario, carpenter bees are much more numerous than they were previously, as it is only in recent decades that they have begun to be noticed by homeowners and pest control operators, despite their presence in the province for at least century (Skandalis et al. 2011).

The Western group also provided evidence for range expansion, but the evidence was not as strong as for the northern group. Two western populations (IA and AR) formed their own distinct genetic group with significant genetic differentiation from the Core group. However, the western populations (IA and AR) did not show decreased levels of allelic diversity, nor did they show signs of inbreeding or recent bottlenecks. One possibility is that range expansion to the west did indeed take place, but significantly earlier than the northern expansion, so that strong genetic signatures of recent range expansion may already be lost. The western portion of the range of *X. virginica* is dominated by prairie landscapes with few large trees, suggesting that nesting substrate would have been limiting until the late 19th century when humans began to construct large numbers of wooden buildings and fences. As a result, the growth of human populations could have facilitated the growth of bee populations and westward range expansion (Skandalis *et al.* 2011). If range expansion was rapid and involved large numbers of individuals, this could have prevented a loss of allelic richness, just as was seen in the westward range expansion of *Colletes hederae* (Dellicour *et al.* 2014).

Conclusions

Xylocopa virginica populations show high levels of genetic differentiation among populations, but in general maintain low levels of inbreeding across their range. This differentiation is likely caused by the philopatric nature of their nesting habits, coupled with the patchy availability of the anthropogenically modified nesting substrate they use. Temperature helps explain genetic variation among populations, which may mean that genetic differentiation of edge populations could be related to local adaptation to more extreme climates. This seems especially likely for the Northern group, which appears to

be part of a recent population expansion, likely taking advantage of increasingly milder winter temperatures at higher latitudes. *Xylocopa virginica* indeed appears to be one of the rare "anthrophilic" species that can thrive in association with humans. This adaptability will serve them well, as environments will no doubt continue to be modified into the foreseeable future.

Acknowledgements

We would like to thank the many people who helped collect specimens or allowed us to collect bees on their property, specifically Michael Hutchinson, Dr. Russell Misell, Moni Hayne, Dr. Amber Tripodi, Morris Brown and Dwight Williams, along with field assistants Connie Vickruck and Wes Lesco. Brock Harpur and several anonymous reviewers provided valuable comments on earlier versions of the manuscript. This research was supported by Natural Sciences and Engineering Research Council (NSERC) and Ontario Graduate Scholarships to JLV and an NSERC Discovery grant to MHR.

Data accessibility

Microsatellite genotypes and population information can be found in the Dryad Digital Repository: doi:10.5061/dryad.gj14q

Author contributions

JV and MR designed the experiment. JV collected and genotyped specimens and analyzed the data. MR provided equipment and reagents. JV and MR wrote the manuscript.

Table 2.1. Collection locations and coordinates as well as genetic characteristics for 328 female *Xylocopa virginica* collected and genotyped at 9 microsatellite loci. All collection locations were in the eastern United States with the exception of the Niagara location, in Ontario, Canada. Latitude and longitude are reported in decimal degrees. **N**=Number of individuals collected at that location. **N_{AR}**=Allelic richness rarefied over the smallest sample size in the dataset, **N_p**=Number of private alleles, **Ho**=Observed heterozygosity, **He**=Expected heterozygosity, **F_{IS}**=Inbreeding coefficient. **Ne**=Effective population size (95% confidence interval).

Region, State/Province	Collection code	Latitude	Longitude	N	N_{AR}	N_P	Ho	He	F_{IS}	Ne
St. Catharines, Ontario	ON	43.12250	-79.23694	29	3.84	1	0.49	0.55	0.12	18 (7-29)
Fairfield, Iowa	IA	41.05266	-92.02553	13	5.11	-	0.66	0.66	-0.003	29 (9-1232)
Benton County, Arkansas	AR	36.22163	-94.48435	20	6.1	3	0.71	0.72	0.01	50 (19-137)
Clayton, Ohio	OH	39.83507	-84.25919	10	5.89	1	0.74	0.73	-0.03	∞
Lexington, Kentucky	KY	38.01659	-84.50139	15	6.18	4	0.65	0.72	0.09	36 (12-149)
Portland, Tennessee	TN	36.63610	-86.57309	16	5.73	3	0.70	0.76	0.07	19 (6-44)
Palmyra, Pennsylvania	PA	40.30850	-76.59100	31	6.31	1	0.73	0.78	0.06	63 (23-109)
Beltsville, Maryland	MD	39.04500	-76.87750	20	6.44	2	0.77	0.74	-0.04	285 (75-∞)
Richmond, Virginia	VA	37.53833	-77.47694	20	5.96	1	0.72	0.72	0.003	37 (14-76)
Raleigh, North Carolina	NC(R)	35.60805	-78.56861	24	6.03	2	0.75	0.73	-0.03	27 (11-43)
Greensboro, North Carolina	NC(G)	36.07194	-79.84638	32	6.26	-	0.73	0.77	0.06	30 (13-41)
Goose Creek, South Carolina	SC(G)	33.05416	-79.95416	26	6.07	-	0.72	0.72	0.002	52 (22-82)
Kings Mountain, North Carolina	NC(K)	35.18305	-81.41166	20	5.96	-	0.78	0.75	-0.04	28 (11-49)
Spartanburg, South Carolina	SC(S)	34.92166	-81.96222	24	6.19	1	0.79	0.77	-0.02	37 (15-69)
Juliette County, Georgia	GA	33.05500	-83.72694	6	4.56	-	0.72	0.65	-0.12	∞
Tallahassee, Florida	FL	30.45500	-84.25333	22	6.9	2	0.75	0.79	0.04	31 (12-52)

Table 2.2. Characteristics of the nine microsatellite loci amplified in 328 females of *Xylocopa virginica.* Allele size range indicates DNA fragment length. **N_A**=Number of alleles, **Ho=**Observed heterozygosity, **He=**Expected heterozygosity, **F_{ST}**=Fixation index (Weir and Cockerham 1984), **G'_{ST}**=Genetic differentiation measure (Hedrick 2005). All F_{ST} and G'_{ST} values are significantly different from zero ($P<0.0001$).

Locus	**Allele size range**	**N_A**	**Ho**	**He**	**F_{ST}**	**G'_{ST}**
XV3	221-265	11	0.640	0.769	0.090	0.305
XV7	292-345	15	0.703	0.691	0.106	0.292
XV23	333-424	21	0.797	0.803	0.077	0.301
XV24	180-218	13	0.668	0.641	0.076	0.152
XV27	212-257	18	0.738	0.822	0.059	0.202
XV30	285-310	10	0.623	0.606	0.072	0.125
XV39	216-265	17	0.755	0.708	0.108	0.321
XV42	439-472	12	0.788	0.771	0.080	0.271
XV43	189-261	16	0.713	0.698	0.086	0.221
Mean across all loci		**14.8**	**0.713**	**0.723**	**0.084**	**0.232**

Table 2.3. Analysis of molecular variance (AMOVA) for 16 sample populations. In the upper panel (a), data was partitioned into three groups, the Northern, Western, and Core groups. In the middle panel (b), data was partitioned into four groups, Northern, Western, Eastern Core, and Western Core. The final panel (c) is only the Core populations, split into Eastern and Western Core. Bold text indicates significance at P<0.00001 for each level of variation.

a) All populations, *K*=3

Source of variation	d.f.	Sum of squares	Variance components	Percent of variation
Among groups (K=3)	2	88.202	0.33942	**9.13**
Among populations within groups	13	95.267	0.10181	**2.74**
Within populations	640	2096.118	3.27518	**88.13**
Total	655	2279.587	3.71641	**100.00**

b) All populations, *K*=4

Source of variation	d.f.	Sum of squares	Variance components	Percent of variation
Among groups (K=4)	3	101.988	0.20063	**5.63**
Among populations within groups	12	81.482	0.08851	**2.48**
Within populations	640	2096.118	3.27518	**91.89**
Total	655	2279.587	3.56433	**100.00**

c) Core only, *K*=2

Source of variation	d.f.	Sum of squares	Variance components	Percent of variation
Among groups (K=2)	1	0.516	0.00001	0.00
Among populations within groups	11	5.659	0.00037	0.07
Within populations	519	259.31	0.4997	**99.93**
Total	531	265.485	5.0001	**100.00**

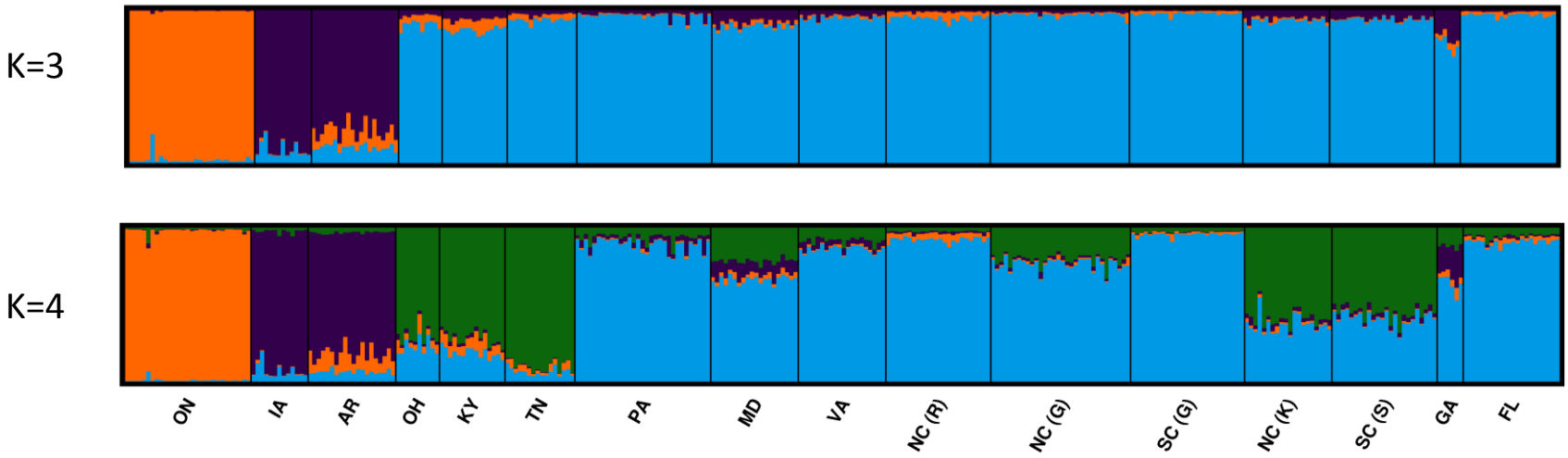
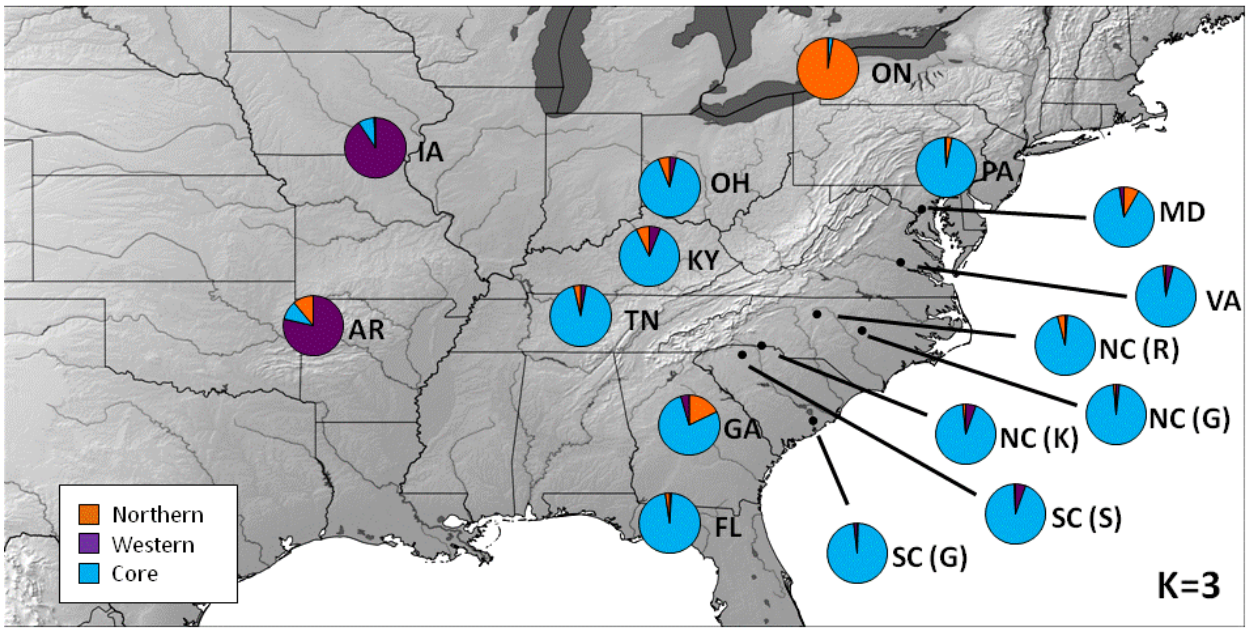


Figure 2.1. Population membership for all 16 populations of *Xylocopa virginica* as computed by Structure for *K*=3 and *K*=4 from 9 microsatellite loci. Each vertical line represents the probability of an individual's genome that can be assigned to a particular population denoted by different colours. Probabilities were calculated as means over 10 runs for each *K* (*K*=3 and *K*=4) in Structure and visualized with Clumpak. Orange bars denote membership to the Northern group, purple to the Western group. When *K*=3 blue denotes membership to the core group. When *K*=4, green bars indicate Western Core and blue Eastern Core groups.

A)



B)

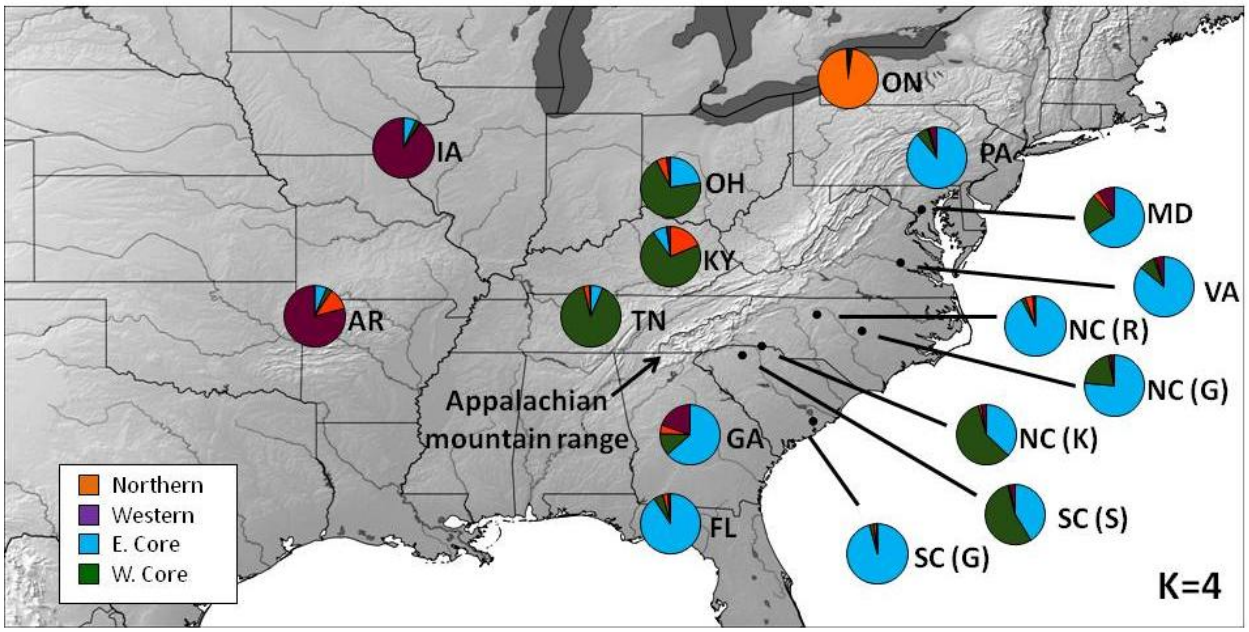


Figure 2.2. Map of sample populations displaying pie charts of mean group membership for each sampled population of *Xylocopa virginica*. Proportions presented in each pie chart represent the mean probability that individuals belong to the Northern, Western, Eastern Core or Western Core groups. Results from Structure analysis based on A) the Evanno (2005) method and B) the Pritchard *et al.* (2000) method.

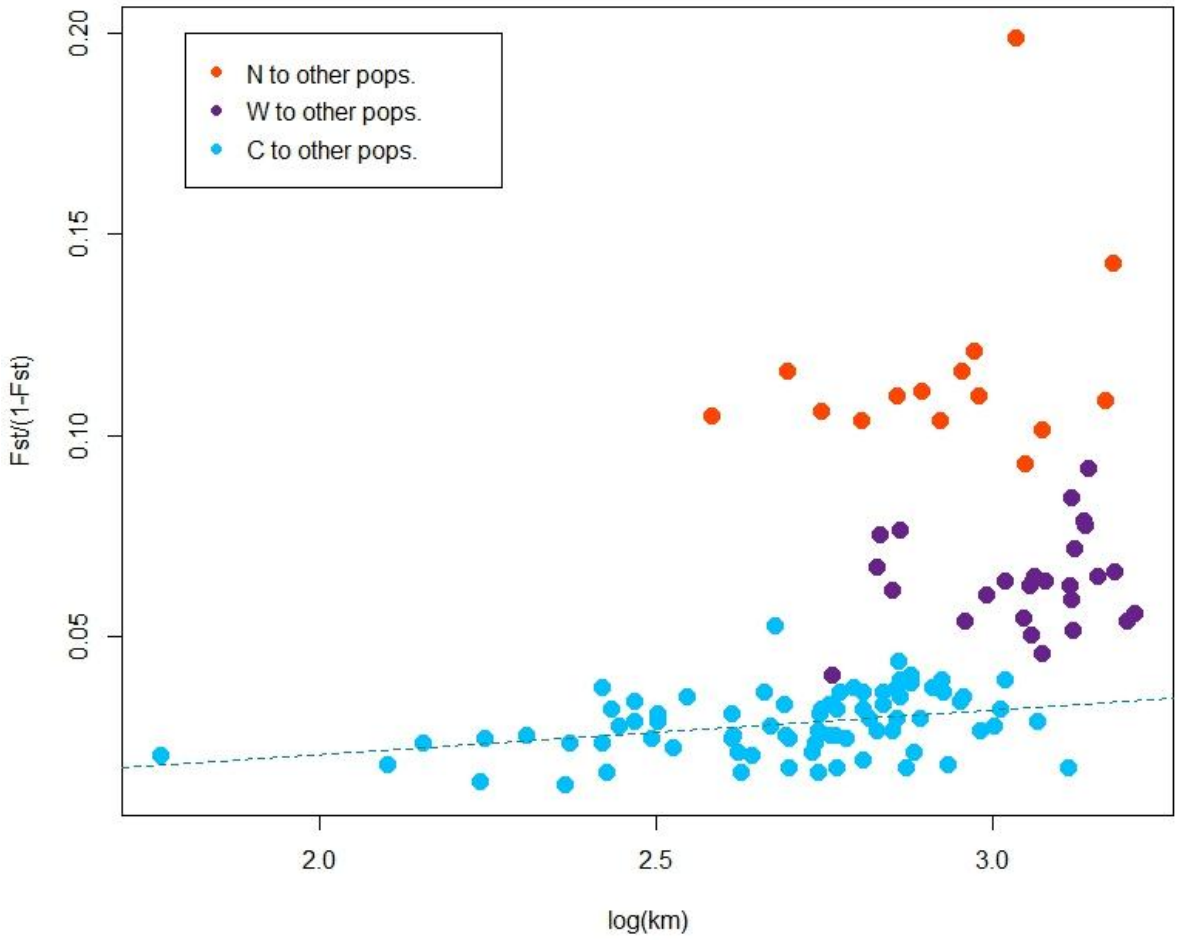


Figure 2.3. Isolation-by-distance among the 16 sample populations of *Xylocopa virginica*. Orange dots represent the comparisons between the Northern group (ON) and the other sample populations, purple dots the comparisons between the Western group (IA and AR) and all other populations, and blue dots the comparisons among populations within the Core group. Regression line drawn for within-group comparisons of the Core group only.

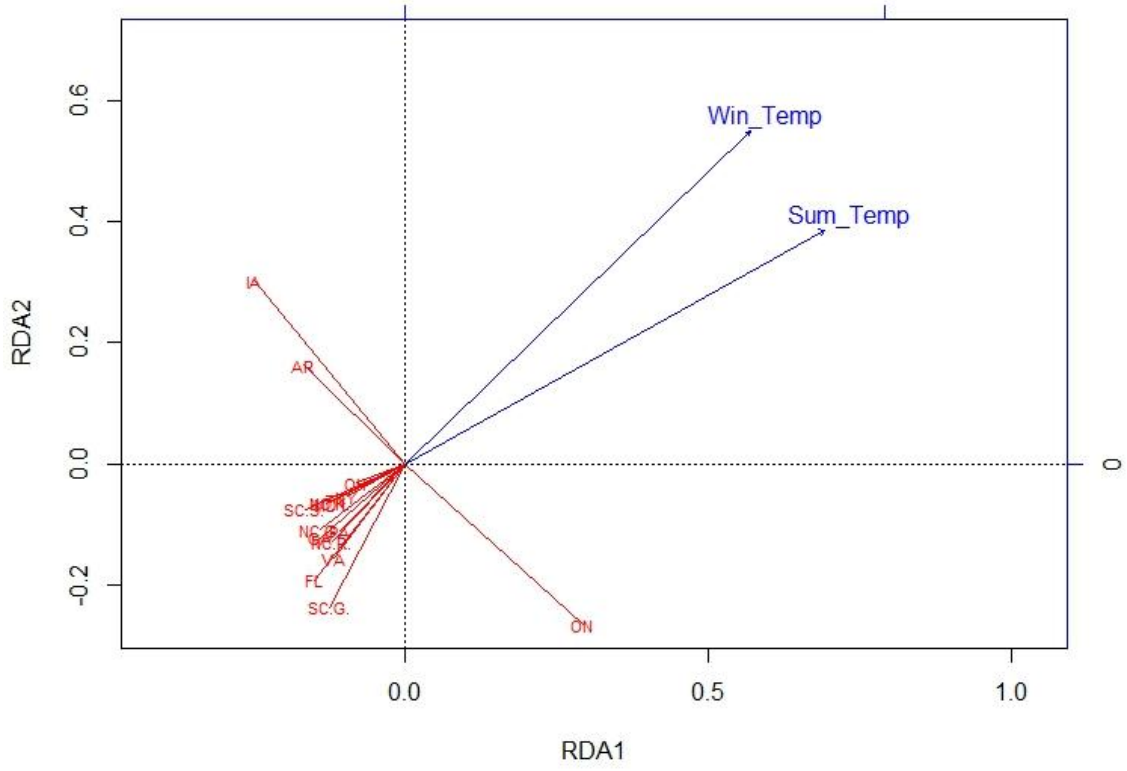


Figure 2.4. Triplot of distance-based redundancy analysis for the significant model which includes mean monthly summer temperature (Sum_Temp) and mean monthly winter temperature (Win_Temp) as environmental explanatory variables (blue vectors). Red vectors represent population genetic differentiation among *X. virginica* populations

Table S2.1. Forty year (1974-2014) monthly average weather data (± standard deviation) obtained from the National Oceanic and Atmospheric Association (ncdc.noaa.gov). All temperature values reported in ^{o}C. Summer comprises the months of April to October, winter from November to March. All values represent mean monthly averages.

		Summer		Winter	
Sample population	**Weather station**	**Temperature (^{o}C)**	**Precipitation (mm)**	**Temperature (^{o}C)**	**Precipitation (mm)**
ON	Ridgeville ON	16.4 ± 5.12	799 ± 386.94	0.7 ± 5.75	692 ± 324.55
IA	Fairfield IA	19.6 ± 4.76	1067 ± 618.89	1.9 ± 6.54	528 ± 380.96
AR	Siloam AR	21.5 ± 4.41	1125 ± 653.27	6.9 ± 5.31	825 ± 613.66
OH	Dayton OH	19.1 ± 4.57	950 ± 490.69	3.3 ± 5.68	738 ± 373.61
KY	Lexington KY	20.3 ± 4.29	1080 ± 589.08	5.6 ± 5.20	909 ± 486.66
TN	Portland TN	21.5 ± 4.14	1094 ± 602.76	7.1 ± 5.07	1077 ± 614.32
PA	Lebanon PA	18.4 ± 4.50	1063 ± 625.64	3.4 ± 5.15	805 ± 445.13
MD	Beltsville MD	20.0 ± 4.57	1003 ± 603.18	5.4 ± 4.87	813 ± 472.86
VA	Richmond VA	21.7 ± 4.15	1024 ± 626.12	7.9 ± 4.62	844 ± 471.05
NC(R)	Raleigh NC	22.3 ± 3.89	1037 ± 640.28	9.1 ± 4.34	922 ± 496.93
NC(G)	Greensboro NC	21.6 ± 3.93	1083 ± 672.80	7.7 ± 4.55	870 ± 448.06
SC(G)	Charleston SC	24.5 ± 3.39	1324 ± 910.83	13.3 ± 3.97	824 ± 553.07
NC(K)	Ninety Nine Islands SC	21.3 ± 3.93	983 ± 593.12	8.4 ± 4.17	981 ± 538.89
SC(S)	Spartanburg SC	22.3 ± 3.75	1011 ± 627.70	9.7 ± 4.03	1047 ± 559.15
GA	Monticello GA	23.0 ± 3.67	936 ± 607.55	10.7 ± 4.24	1019 ± 571.38
FL	Tallahassee FL	25.1 ± 3.20	1500 ± 983.77	14.5 ± 3.87	1113 ± 752.17

Table S2.2. Pairwise measures of genetic differentiation among sample populations. Below diagonal: Pairwise F_{ST} values for all population pairs. Above diagonal: Pairwise D_{EST} values for all population pairs. Significant pairwise differences are based on 9999 permutations in GenAlEx and are indicated by grey shading and boldface text.

	ON	**IA**	**AR**	**OH**	**KY**	**TN**	**PA**	**MD**	**VA**	**NC(R)**	**NC(G)**	**SC(G)**	**NC(K)**	**SC(S)**	**GA**	**FL**
ON		**0.596**	**0.476**	**0.333**	**0.346**	**0.382**	**0.399**	**0.403**	**0.333**	**0.349**	**0.413**	**0.307**	**0.405**	**0.450**	**0.272**	**0.410**
IA	**0.166**		**0.114**	**0.226**	**0.275**	**0.302**	**0.268**	**0.220**	**0.323**	**0.281**	**0.266**	**0.382**	**0.241**	**0.215**	**0.161**	**0.357**
AR	**0.125**	**0.039**		**0.236**	**0.205**	**0.282**	**0.292**	**0.238**	**0.287**	**0.301**	**0.250**	**0.370**	**0.194**	**0.241**	**0.197**	**0.331**
OH	**0.096**	**0.063**	**0.057**		0.008	0.054	**0.086**	**0.081**	**0.092**	**0.107**	**0.103**	**0.109**	0.038	0.048	0.022	**0.136**
KY	**0.099**	**0.071**	**0.051**	0.025		0.057	**0.136**	**0.061**	**0.113**	**0.120**	**0.086**	**0.164**	0.023	**0.135**	0.017	**0.153**
TN	**0.099**	**0.070**	**0.058**	0.030	0.028		**0.100**	**0.127**	**0.132**	**0.158**	**0.081**	**0.123**	**0.069**	**0.087**	0.135	**0.163**
PA	**0.095**	**0.059**	**0.053**	**0.029**	**0.034**	**0.026**		**0.086**	**0.115**	**0.113**	**0.053**	**0.061**	**0.111**	**0.092**	0.028	**0.050**
MD	**0.104**	**0.056**	**0.051**	**0.031**	**0.026**	**0.033**	**0.023**		**0.069**	**0.076**	**0.037**	**0.109**	0.026	**0.058**	0.040	**0.109**
VA	**0.094**	**0.078**	**0.062**	**0.035**	**0.036**	**0.036**	**0.028**	**0.024**		**0.061**	0.031	**0.064**	0.040	**0.083**	0.050	**0.095**
NC(R)	**0.094**	**0.067**	**0.061**	**0.035**	**0.035**	**0.038**	**0.026**	**0.024**	**0.023**		**0.052**	**0.079**	**0.068**	**0.116**	0.020	**0.124**
NC(G)	**0.100**	**0.060**	**0.049**	**0.032**	**0.027**	**0.024**	**0.016**	**0.016**	0.016	**0.018**		**0.083**	0.023	**0.029**	0.039	**0.051**
SC(G)	**0.085**	**0.084**	**0.072**	**0.035**	**0.042**	**0.032**	**0.018**	**0.029**	**0.023**	**0.024**	**0.022**		**0.125**	**0.116**	0.047	**0.040**
NC(K)	**0.104**	**0.059**	**0.044**	0.025	0.021	**0.025**	**0.026**	0.017	0.020	**0.023**	0.014	**0.031**		**0.063**	0.013	**0.138**
SC(S)	**0.108**	**0.052**	**0.048**	0.025	**0.035**	**0.025**	**0.021**	**0.019**	**0.024**	**0.028**	**0.013**	**0.027**	**0.020**		0.066	**0.074**
GA	**0.092**	**0.061**	**0.06**	0.039	0.031	0.050	0.031	0.034	0.037	0.030	0.032	0.034	0.030	0.036		0.031
FL	**0.098**	**0.073**	**0.06**	**0.038**	**0.038**	**0.036**	**0.017**	**0.028**	**0.027**	**0.029**	**0.017**	**0.017**	**0.031**	**0.021**	0.033	

Table S2.3. The influence of latitude and longitude on the population genetic structure of *X. virginica* using GESTE v2.0. (a) posterior probabilities for latitude, longitude and the interaction term separately, (b) posterior probabilities for each model tested. The highest probability model is indicated in bold.

	Factor	**Posterior probability**
a)	Latitude	0.512
	Longitude	0.139
	Latitude x longitude	0.006
b)	Constant	0.423
	Constant, latitude	**0.432**
	Constant, longitude	0.059
	Constant, latitude, longitude	0.079
	Constant, latitude, longitude, latitude x longitude	0.006

Table S2.4. Testing for recent population bottlenecks using Bottleneck software using the two phase model (TPM) with 95% single step mutations and 5% muli-step mutations. Significance based on Wilcoxon tests. None of the sample populations shows signs of a bottleneck as indicated by significant heterozygosity excess.

Sample population	Population	Group	Heterozygosity excess P value
ON	St. Catharines	Northern	0.990
IA	Fairfield	Western	0.875
AR	Benton County	Western	0.850
OH	Clayton	Core	0.285
KY	Lexington	Core	0.875
TN	Portland	Core	0.285
PA	Palmyra	Core	0.455
MD	Beltsville	Core	0.999
VA	Richmond	Core	0.936
NC(R)	Raleigh	Core	0.986
NC(G)	Greensboro	Core	0.936
SC(G)	Goose Creek	Core	0.997
NC(K)	Kings Mountain	Core	0.674
SC(S)	Spartanburg	Core	0.455
GA	Juliette County	Core	0.752
FL	Quincy, Montecello	Core	0.787

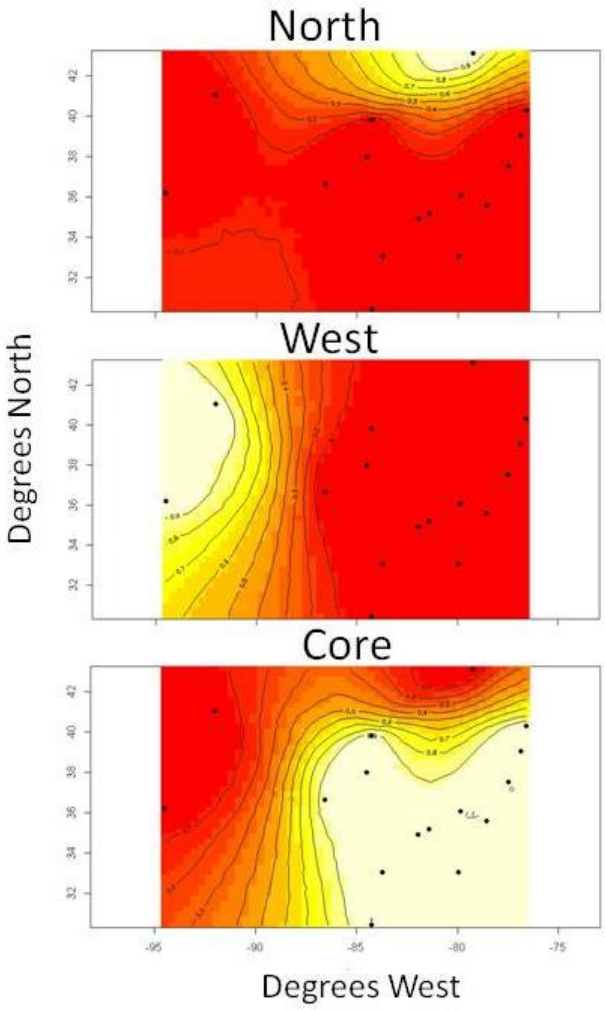


Figure S2.1. Posterior probability of belonging to each group as calculated in Geneland. Axes are latitude and longitude respectively and points represent sample populations. Shading is proportional to the probability of membership to the specified group where white is a high probability and red is a low probability.

Rationale for chapter three

Chapter two demonstrated that despite being linked to anthropogenic disturbance, carpenter bee populations showed high genetic diversity and appear to be expanding their range northward and potentially westward. In general, populations were genetically distinct from one another, a result which we explained through nest philopatry exhibited by *X. virginica* females (Rau 1933; Gerling & Hermann 1978; Skandalis et al. 2011). The first data chapter aimed to understand the genetic structure of *X. virginica* across its range. In this chapter I focus on a single population from Niagara, the northernmost point of the population genetic dataset. Eastern carpenter bees often form small social groups, where females live together for extended periods of time (Gerling & Hermann 1978; Richards & Course 2015). Within these social groups not all individuals are reproductive; instead, they appear to form dominance hierarchies. (The nature of these hierarchies will be explored further in chapter 4). Social interactions among adult females before offspring are provisioned likely impact the dominance hierarchies that form within nests.

An important component of social groups is the ability to recognize conspecifics as members of the group. In this chapter I investigate whether *X. virginica* females use nestmate or kin recognition when interacting with conspecifics inside the nest. In 2011, a pilot experiment placed overwintering females into observation nests with hopes to observe how dominance hierarchies form in the spring. The initial diameter of the observation nest tunnels was too narrow and nests were rejected by females, but preliminary observations showed that many interesting behaviours took place inside nests

during the nestmate provisioning phase. Many of these behaviours had not been seen in the previous nestmate study of Peso and Richards (2010a) which was conducted in circle tubes, indicating that context can affect the behaviours displayed by interactants. Observation nests were redesigned in 2012 to allow females to interact more freely inside nests and are the subjects of chapter three. By understanding how recognition takes place in the nest, I will be better able to understand how reproductive queues form in eastern carpenter bee colonies.

Chapter 3: A test of kin and nestmate recognition in the eastern carpenter bee: Familiarity matters more than family

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Author contributions: JLV and MHR designed the experiment. JLV collected observational data and genotyped specimens. JHV and MHR analyzed the data. MHR provided equipment and reagents. JLV wrote and MHR edited the manuscript.

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Introduction

Broadly speaking, recognition is the ability of one individual to consistently identify another (Sherman et al. 1997) and has been documented across a wide variety of insects, fish, birds, and mammals (Gamboa et al. 1986a; Holmes 1986; Breed & Url 2011; Breed 2014). Animals that live in social groups must frequently decide whether the conspecifics they encounter are part of their established group or are outsiders. Failing to discriminate against non-members may result in the depletion of limiting food or nesting resources (Boff et al. 2015), increased parasitism (Kreuter et al. 2012), the killing of immature or juvenile offspring, and even supersedure of dominant individuals (Hogendoorn & Velthuis 1995; Hogendoorn 1996), outcomes that can decrease the fitness of the individuals in the group. In contrast, cooperation among group members can increase their fitness by increasing ergonomic efficiency and offspring survival, and by decreasing parasitism (Clutton-Brock 2002).

Two types of recognition mediate how individuals make decisions when encountering conspecifics: the ability to recognize kin and the ability to recognize individuals based on learned cues. Kin recognition occurs when a cue-receiver behaves differently towards genetically related versus unrelated cue-bearers (Sherman et al. 1997). In kin recognition, the cue-receiver must be able to recognize shared genetic traits in the cue-bearer that are identical by descent without previous contact with one another. Individual or nestmate recognition (recognition not based on kinship cues) occurs when a cue-receiver behaves differently towards known or unknown cue-bearer, following a period of contact or a series of interactions in which interactants become familiar to one another (Sherman et al. 1997; Dale et al. 2001). For animals that spend their lives in kin

groups with closed membership (new members are not accepted from outside sources), nestmate and kin recognition may, in effect, be one and the same because group members are also kin. In contrast, when group membership is fluid (individuals may join or disperse from the group), groups may include kin and non-kin, as well as familiar and unfamiliar individuals. The ability to distinguish kin from familiar but unrelated individuals should be especially important in societies where some individuals forgo reproduction to help others raise offspring, as the indirect fitness benefits often ascribed to helping can only be obtained by directing help towards related individuals (Hamilton 1964).

To fully differentiate between kin and nestmate recognition, it is necessary to compare recognition behaviour among four potential types of interactants: related and familiar, related and unfamiliar, unrelated and familiar, and unrelated and unfamiliar (Table 1). Predictions about how same-sex nestmates or kin should behave towards one another are based on the underlying assumption that increased cooperation promotes group cohesiveness and the average fitness of group members, while increased aggression among nestmates decreases fitness (Breed 2014). If recognition is based exclusively on relatedness as a cue, then previous experience will not influence behavioural interactions, so cooperation should be more frequent between related individuals, and aggression should be more frequent between unrelated individuals. Similarly, if recognition is based exclusively on learned familiarity, then cooperation should be more frequent between familiar individuals, and aggression should be more frequent between unfamiliar individuals. When social groups contain a mix of related and unrelated individuals, then both kin and nestmate recognition could operate, and

behavioural interactions could be influenced by the relative strengths of kin and nestmate recognition cues. For instance, if individuals are more influenced by relatedness than by familiarity, we would predict more cooperation and less aggression between related, unfamiliar interactants than between unrelated, familiar interactants (Table 1).

In social insects, group members typically live together in a shared nest, and related females cooperate in food acquisition, nest maintenance, and rearing of offspring (Michener 1974). Empirical examples of nestmate recognition are pervasive in social insects, including obligately eusocial wasps (Gamboa et al. 1986a, 1986b), primitively eusocial sweat bees (Soro et al. 2011), ants (Errard 1994; Rosset et al. 2007), and facultatively social carpenter bees (Peso & Richards 2010a). Even non-social bees can be capable of nestmate recognition (Flores-Prado et al. 2008). In most cases, cuticular hyprocarbon profiles are used to distinguish nestmates from non-nestmates (Gamboa et al. 1986a; Van Zweden et al. 2010; Nunes et al. 2011), however visual cues have been implicated in individual recognition in *Polistes fuscatus* wasps (Tibbetts 2002).

Initial evidence for kin recognition came from the eusocial sweat bee *Lasioglossum zephyrum,* in which workers can discriminate among conspecifics of varying degrees of relatedness even without previous contact with one another (Greenberg 1979, 1988). Gregarious cockroaches also demonstrate the ability to discriminate kin based on heritable hydrocarbon profiles (Lihoreau et al. 2016). In contrast to recognition based on familiarity, empirical evidence for kin recognition is quite rare (Boomsma & D'Ettorre 2013; Breed 2014). One reason for this is that in many social insects, colonies contain significant numbers of unrelated individuals (Abrams & Eickwort 1981; Kukuk et al. 2005; Leadbeater et al. 2010). This insight invalidates any

assumptions that nestmate recognition is, in effect, kin recognition, and that discrimination against non-nestmates is equivalent to discrimination against non-relatives. Moreover, even if a species is capable of kin recognition, its behaviour towards related and unrelated nestmates may very well be modified by learning, so that unrelated but familiar nestmates come to be treated similarly to kin. In *Lasioglossum zephyrum*, workers use both kin (Greenberg 1988) and nestmate recognition (Buckle & Greenberg 1981) when interacting with nestmates. Female stingless bees (*Frieseomelitta varia*) use nestmate recognition cues even when kin recognition cues are available (Nunes et al. 2011). Newly emerged *F. varia* workers have hydrocarbon profiles similar to their relatives, yet are easily transplanted and accepted into new colonies. However, after as little as twenty minutes females acquire the specific hydrocarbon profile of their home nest and are then rejected by workers from their natal nests (Nunes et al. 2011).

To answer questions about the evolution of group living, the best model organisms are those that maintain a flexible social repertoire. Facultatively social species display more than one behavioural state, living either solitarily or in social groups, and in some cases, groups may be composed of varying proportions of related and unrelated individuals. Such behaviourally flexible species could use either kin or nestmate recognition (or both) in maintaining social cohesion, providing opportunities for assessing the influence of relatedness and familiarity on nestmate interactions in natural settings.

An ideal model for studying interactions among related and unrelated nestmates is the eastern carpenter bee, *Xylocopa virginica*, which can nest solitarily or socially. Siblings overwinter together in their natal nests, but many females disperse to join new

colonies during the breeding season (Peso & Richards 2010b; Richards & Course 2015). As a result, social females interact with both related and unrelated nestmates inside the nest, for periods as short as hours or days or as long as months (including the winter months). Adult bees can discriminate between nestmates and non-nestmates (Peso and Richards 2010a). In circle tube assays, same-sex pairs of both females and males that had spent the previous night in the same nest were more tolerant and less aggressive to each other than pairs of bees from different nests. Thus bees treated nestmates as familiar individuals after as little as 24 hours in the same nest. Demonstrating that familiarity alone does not guarantee tolerance, in female-male pairs, aggression was more frequent among nestmates than non-nestmates. Since adult carpenter bees frequently leave one colony to join another (Peso and Richards 2010b, Richards and Course 2015), these results demonstrated that in eastern carpenter bees, adult females and males are capable of context-dependent nestmate recognition based on familiarity, influencing social interactions within and between the sexes. Whether or not kin relationships also influence recognition and subsequent behaviour remained an open question.

Objectives

In the current study, our primary objective was to investigate whether nestmate recognition in eastern carpenter bees is influenced by genetic cues that would indicate kin recognition, or is based solely on learned cues and familiarity. Our approach was to examine recognition behaviour during the nestmate provisioning phase of colony development in early spring. During this early phase of the colony cycle prior to egg-laying, social females feed adult nestmates, suggesting that feeding behaviour is

involved in establishing dominance hierarchies and reproductive queues that structure reproductive skew in the latter, brood provisioning phase of the colony cycle (Richards & Course 2015). As feeding of adult nestmates exemplifies cooperation among group members and aggression exemplifies conflict, our second objective was to examine feeding, aggressive and other behavioural interactions during the period when dominance hierarchies are formed. During the nestmate provisioning phase of the colony cycle, females naturally interact with nestmates representing all possible combinations of relatedness and familiarity (Table 3.1), which allowed us to investigate the extent to which *X. virginica* females use kin recognition, nestmate recognition based on familiarity, or both in interactions among colony-mates. We predicted that if females use kin recognition to discriminate among individuals they encounter in their own nests, cooperative behaviour should be more frequent and aggression should be less frequent among related than unrelated individuals (Table 3.1). Conversely, if nestmate recognition is the predominant method of recognition, cooperation should be more frequent and aggression frequent among familiar than unfamiliar individuals. If both kin and nestmate recognition are used, then related, familiar bees should be the most cooperative and least aggressive, while unrelated, unfamiliar bees should be the least cooperative and most aggressive (Table 3.1).

Methods

Seasonal phenology and nesting biology of Xylocopa virginica

In southern Ontario, the colony cycle of *X. virginica* begins in April, when adult bees awaken from hibernation. First males and then females emerge from their nests

when daytime temperatures first reach 20^{o}C. For females, a period of nestmate provisioning ensues, during which foragers bring pollen back to the nests to feed to adult nestmates (Richards & Course 2015). The nestmate provisioning period is followed by the brood provisioning period, which lasts from mid-May until mid-July, after which adult bees mostly remain inside their nests. Brood typically eclose from August to September, but remain inside their natal nests over the winter. Adult bees huddle together at the ends of their burrows throughout the winter (Figure 3.1), so natal nestmates spend at least eight months (September to April) in intimate contact.

Female eastern carpenter bees can nest solitarily or socially (Peso & Richards 2010b; Richards 2011; Richards & Course 2015). Social colonies of *X. virginica* are small, typically comprising 2 to 8 adult females during the brood provisioning phase (Richards 2011). Prior to emergence from hibernation, nest groups are comprised of natal nestmates produced by one to several mothers. However, with the onset of the nestmate provisioning period, many females disperse away from their natal nests to join other colonies (Peso & Richards 2010b; Richards & Course 2015). By the time that brood provisioning begins, social groups may be composed of a mix of nestmates and non-nestmates, some of which have had only days or weeks of prior contact.

Observation nest set-up

In March 2012, four large cedar boards that contained 21 overwintering nests of *Xylocopa virginica* were removed from an arbour on the Brock University campus in St. Catharines, Ontario, Canada. Nests were opened outside on 30 March 2012 when the temperature was below 4^{o}C, ensuring that bees were inactive and could be easily handled.

Each nest was carefully opened using an electric plane to expose overwintering bees. Opening nests at this temperature does not disrupt hibernation.

All bees were removed from their nests for morphometry and to collect a tissue sample for DNA analyses. We measured head width (the widest part of the head including the compound eyes), because the vast majority of interactions between bees take place head to head, and a female's head is the first part of her body to enter a nest. We also measured intertegular width (the distance between the tegulae across the thorax); this measurement proved to be non-significant in all analyses, so these results are not presented in this study. The last tarsus of the left metathoracic leg was removed with microscissors and placed in 100% redistilled ethanol for later genetic analysis. Each bee was marked with a unique two colour combination using Testor's enamel paint for later identification in the field. Bees were categorized as familiar if they were found overwintering in the same nest and as unfamiliar if they had overwintered in different nests. Note that these definitions differ from those used by Peso and Richards (2010a) in the previous study.

After handling, bees were placed into the tunnels of observation nests in the same order in which they were found in their overwintering nests. Five bees (3 female and 2 male) that were dead, and two females that were damaged in the process of opening the winter nests were not placed in observation nests. In total 154 bees (92 females and 62 males) were placed in observation nests.

Each observation nest contained one predrilled nest entrance with a linear tunnel 40 cm in length, 20 cm on each side of the nest entrance (Supplemental Figure S3.1). The nest entrance and the tunnels were semi-circular with a 9 mm radius. After bees

were placed in their observation nest, a piece of clear Plexiglas was placed over top, secured with screws, and then sealed around the edges with all-weather caulking. A piece of particle board was hinged over top of the Plexiglas to darken the nests and was only raised when the nests were under observation. Observation nests were secured in frames and placed at the edge of a field near the Brock University campus (43.1243, -79.2331 decimal degrees) to allow bees to emerge naturally in the spring. To encourage dispersing females to stay in the observation area, 19 additional, empty nests were provided, for a total of 40 available observation nests.

Behavioural observations

Behavioural observations took place during the 2012 nestmate provisioning phase, from 4 to 25 May 2012 from 0830–1630 hours on days without rain when the temperature was above 18°C. Prior to observations each morning, each nest was opened and the number of males and females inside recorded. We report observations of both females and males, but all analyses pertain to interactions among females.

Behavioural observations focussed on female bees returning to nests. When a female returned to and entered a nest she was designated as the focal individual, the particle board cover was lifted, and all behaviours of the focal bee and any bees interacting with her were recorded for two minutes. The identity of the focal female, whether or not she was carrying pollen on her scopae, the identities of all interactants, and the nest and time, were recorded. Occasionally a non-focal bee would have its back to the Plexiglas, or the focal bee would interact with a new, unmarked bee from the surrounding population already inside the nest. In either case the second bee was

recorded as unidentifiable. After two minutes the particle board cover was replaced so as not to disrupt the bees excessively.

In total, 352 two-minute observation periods were recorded between 4 and 25 May 2012. A detailed ethogram of the 19 behaviours recorded in these two-minute bouts across all bees is presented in Table 3.2. Behaviours were grouped into four categories for further analysis: feeding, aggression, other interactions (pass, head to head touch, attempted pass), and individual behaviours.

Genetic relationships among females

Genomic DNA was extracted from the tarsal segment collected during initial marking using 10% Chelex solution and proteinase K, as described by Casquet et al (2012). Each genomic sample was amplified at 9 microsatellite loci previously described by Vickruck (2015). Loci were amplified individually in 15µl PCR reactions with 40-70 ng of DNA, 1 unit Standard *Taq* (New England Biolabs), 1x Thermo Buffer (New England BioLabs), 0.2 mM dNTPs, 0.2 µM forward primer, and 0.2 µM reverse primer. Forward primers contained a flourophore (either 56-FAM or HEX), to allow for fragment size calling. PCR conditions were as follows: 95°C for 3 min, 40 cycles of 94°C for 30 sec, 55 or 52°C for 30 sec, 72°C for 30 sec. PCR reactions for loci XV24, XV27, XV7 and XV30 used annealing temperatures of 55°C and loci XV39, XV42, XV43and XV3 used annealing temperatures of 52 °C. Locus XV23 used a touchdown procedure where during the first cycle the annealing temperature was 65°C and for the first twenty cycles decreased by 0.5°C per cycle. During the last 20 cycles the annealing temperature remained at 55°C. Each PCR run contained a negative control as well as two previously

run positive controls to account for any variation among runs. PCR products were visualized using a 3730xl DNA Analyser (Applied Biosystems) at the Peter Gilgan Centre for Learning and Research in Toronto, Canada, and genotyped using GeneMapper v3.5 (Applied Biosystems). All allele calls were double checked by hand.

Relatedness (*r*) calculations were conducted in GenAlEx 6.5 (Peakall and Smouse 2012) using the method of Queller and Goodnight (1989). Individuals with missing genotypes at three or more loci were not included in relatedness calculations. Life-for-life values of *r* range between 0 and 1, where 0 means two individuals share no alleles at any loci and 1 indicates two individuals that are identical (Queller & Goodnight 1989). Regression estimates of *r* based on the formula by Queller and Goodnight range from -1 to 1 (Queller and Goodnight 1989). Hymenoptera are haplodiploid, meaning that full sisters (female offspring produced by a singly mated mother) are related by $r = 3/4$. Negative values indicate unrelated individuals. Full sister relatedness assignments were also assessed using Kingroup (Konovalov et al. 2004) which allowed us to test the hypothesis that pairs of females were full sisters versus unrelated pairs. Pairs of females that were significantly more likely to be full sisters were categorized as related, pairs that were not significantly were categorized as unrelated.

Data Analysis

Statistical analyses were conducted in R version 3.2.1 running in RStudio 0.98.1056. Parametric statistics were used whenever analyses conformed to the assumptions of the normal distribution, otherwise non-parametric tests were used. Data in figures are presented as medians and upper and lower quartiles unless otherwise noted.

Behavioural events during the nestmate provisioning period and the influence of pollen foraging on nestmate interactions were analyzed using all behavioural data collected (98 bees, 667 behaviours from 352 observation periods). To examine the effects of body size and relatedness on nestmate interactions we used only females for which both individuals could be identified and for which we had genotypic data (62 females, 59 unique pairs of bees). Pairs of interacting females did not occur in equal proportions with respect to relatedness (full sisters or not) and familiarity, and the category of related but unfamiliar females contained only three pairs (Table 3.3). To tease apart the effects of kin and nestmate recognition, we tested for effects of familiarity on behaviour by comparing unrelated, familiar pairs to unrelated, unfamiliar pairs. We tested for effects of relatedness on behaviour by comparing related, familiar pairs to unrelated, familiar pairs.

We used a MANOVA to simultaneously test the influence of relatedness (Queller and Goodnight's *r*), familiarity (familiar vs. unfamiliar), whether or not females returned to the nest with pollen, the difference in head width (body size) between the interacting bees and the identity of the focal female, on a matrix of three response variables (frequency of feeding, passing and aggressive behaviours). We first created summary files containing the interactions of all observed pairs of females for which we could identify both interactants and for which microsatellite genotypes were available to calculate a pairwise relatedness value. Wilks λ was used to calculate F statistics for relatedness and familiarity in the MANOVA analysis. Body size differences were calculated as the head width of the focal female minus the head width of the other bee.

Results

Behavioural events during the nestmate provisioning period

We recorded 667 behaviours from 352 two-minute observation periods from 4 to 25 May 2012. On 57/667 occasions, the second bee in the interaction could not be identified. The number of behaviours observed per 2 minute observation period ranged from 1 to 8 (mean 1.8±1.0 behaviours/trial, median=2.0). The number of bees per nest each day fluctuated and decreased over the course of the nestmate provisioning period (Supplementary Figure S3.2).

Sixty of the 667 behaviours were performed by males entering the nest. Of these 60 behaviours, one was a behaviour associated with feeding (receive beg), four were aggressive behaviours, seven were other interactions (all passes), and 48 were individual behaviours; frequencies for male behaviours are presented in Table 3.2. No male was ever observed being fed or feeding another bee in the nest.

Interactions were observed among 59 pairs of female bees in which both interactants could be identified and genotyped. Aggressive and feeding behaviours exhibited different temporal patterns. Aggression by or toward the focal female was observed over the entire nestmate provisioning period (starting on 5 May) and became more severe over time, with biting and C-posturing observed from 9 to 18 May and females being ejected from nests from 12 to 25 May (Supplemental Figure S3.3). Feeding behaviours showed a very different pattern. Feeding behaviour was first observed on 14 May, a few days after aggression had escalated to the level of biting and C-posturing. Once feeding behaviour began, it was a daily occurrence until the end of the

nestmate provisioning period (Supplemental Figure S3.3). Other interactive behaviours involving focal females were observed over the entire observation period and had a unimodal distribution, with the highest number of behaviours taking place on 19 May (Supplemental Figure S3.4). Females were observed performing individual behaviours throughout the entire nestmate provisioning period. The number of individual behaviours increased as the season progressed, with the highest number of individual behaviours taking place on the last observation day, 25 May (Supplemental Figure S3.4).

The influence of pollen foraging and body size on nestmate interactions

Whether or not a focal female was carrying pollen when she returned to the nest had a significant effect on behavioural interactions (Table 3.4 and Supplementary Table S3.1). Not surprisingly, feeding behaviours were more frequent when females returned with pollen and aggressive interactions were more frequent when females returned without pollen (Table 3.4). Other interactions took place at similar frequencies regardless of whether a female was bringing pollen back to the nest. Bees returning to the nest without pollen performed more individual behaviours (Table 3.4).

Females receiving food had significantly larger head widths than bees doing the feeding (Figure 3.1). There was no difference in head width between bees performing other interactive behaviours, or between bees engaged in aggressive interactions (Figure 3.1).

The influence of relatedness and familiarity on behavioural interactions

Of the 59 interacting pairs of females for which both genotypic and familiarity data were available, 23 pairs were familiar and 36 were unfamiliar (Table 3.3). Based on

Kingroup assignments, 11 pairs were full sisters and 48 were unrelated (Table 3.3). Mean relatedness (Queller and Goodnight) among interacting pairs of females across the observation period was $r = 0.15 \pm 0.36$.

Among unrelated females, feeding behaviours were more frequent in familiar than unfamiliar pairs, while aggressive interactions were more frequent among unfamiliar than familiar pairs (Table 3.5). No significant differences were seen in the number of feeding, aggressive or other behaviours among familiar related or familiar unrelated females (Table 3.5). MANOVA results showed that there were two significant predictors of behaviour of interacting pairs of females: whether or not the focal female was carrying pollen when she returned to the nest and whether interactants were familiar or unfamiliar (had overwintered together). Relatedness of interactants was not a significant predictor of behaviour among interacting pairs of females (Table 3.6).

Discussion

The influence of relatedness and familiarity on behavioural interactions

Simultaneous tests of relatedness and familiarity showed that familiarity, but not relatedness, influenced behavioural interactions among female carpenter bees during the nestmate provisioning phase of the colony cycle, prior to brood production. The finding that recognition occurs primarily through familiarity is in agreement with a previous study (Peso and Richards 2010a), although the definition of familiarity differed. In our experiment, some pairs of females that were classified as unfamiliar (since they did not overwinter together) would have been classified as familiar by Peso and Richards (because they spent the night in the same nest). Many pairs of bees in the study by Peso

and Richards (2010a) probably were natal nestmates that had overwintered together and remained in the same nest. In the current study, females that relocated to new nests during the nestmate provision phase were considered to be unfamiliar, but would have been considered familiar by Peso and Richards (2010a) as long as they had been together for at least 24 hours. Thus Peso and Richards detected discrimination between nestmates and non-nestmates even though the design of their study would have obscured these differences. Taken together, our two studies suggest that carpenter bees learn the identities of natal and overwintering nestmates during the winter, but can also learn the identities of new nestmates that join colonies in early spring. Subsequently, females behave differently to more familiar and less familiar individuals. It seems likely that recognition in *X. virginica* occurs along a continuum, and bees are capable of distinguishing among very familiar individuals, somewhat familiar individuals and unfamiliar individuals. It would be very interesting to see how total time of association and time since last association affect behavioural interactions among *X. virginica* females.

One likely mechanism of nestmate identification in eastern carpenter bees is through cuticular hydrocarbons, but there is mixed evidence as to whether hydrocarbon signals relay information about kin or nestmate status. Cuticular hydrocarbon profiles may be heritable (Greenberg 1979; Adams 1991; Breed et al. 1995), but their composition is highly variable, and may change in response to environment (Downs & Ratnieks 1999; Buczkowski & Silverman 2006) and diet (Liang & Silverman 2000). Studies in Hymenoptera have shown that individuals' cuticular hydrocarbon profiles may be an acquired, nest-specific chemical signature, which is used in recognizing conspecific

nestmates (Breed et al. 1995; Nunes et al. 2011). Chemical profiles likely reveal more information than individual identity or colony affiliation. In the orchid bee, *Euglossa melanotricha*, cuticular hydrocarbon profiles actually reflected reproductive status in social nests (Andrade-Silva & Nascimento 2015). Further research into individual hydrocarbon profiles of nestmates and non-nestmates will greatly further our understanding of individual recognition for eastern carpenter bees.

Another potential recognition mechanism is through visual cues. A small number of species in the genus *Polistes* use highly variable facial colouration to recognize individuals in the colony (Tibbetts 2002; Sheehan & Tibbetts 2011; Injaian & Tibbetts 2013). It has been suggested that individual recognition is more likely in small social groups (Lihoreau et al. 2016), which could apply to eastern carpenter bees. Since the faces of *X. virginica* females are entirely black and lack the variation predicted necessary for individual identification by this mechanism (Sheehan & Tibbetts 2010), facial recognition likely did not influence female-female interactions.

The role of spring behaviour in shaping dominance hierarchies within nests

Behavioural interactions such as those examined here, likely play a role in the eventual dominance status of females in social nests for eastern carpenter bees. One of the original goals of this study was to follow females from the nestmate provisioning phase to the brood provisioning phase to observe how spring behaviour was related to final dominance rank. Unfortunately, despite the success of the observation nests in early spring, most females abandoned their observation nests during the brood provisioning phase. This seemed to be because the Plexiglas surface covering the burrow made it

difficult to build brood cell partitions. However, our study does clearly indicate that adult females frequently feed each other for a period of several weeks prior to the onset of brood provisioning. To our knowledge, an extensive nestmate provisioning phase in which foragers bring pollen to the nest to feed to adult nestmates is a novel aspect of colony development in social insects. In most social bees, it would be assumed that if females are regularly bringing pollen to their nests, then the pollen is destined to become larval provisions.

Despite the abandonment of the observation nests by most bees, the timing of aggressive and feeding behaviours during the nestmate provisioning period provides clues to their role in about the development of dominance hierarchies within colonies. In many social insects aggression is more frequent during dominance hierarchy formation or change, but decreases after the hierarchy has stabilized (Monnin & Peeters 1999; Arneson & Wcislo 2003; Cuvillier-Hot et al. 2004; Hoffmann & Korb 2011). We observed aggressive behaviours throughout the nestmate provisioning period, suggesting that it takes several weeks for dominance hierarchies to stabilize. Feeding behaviours, however, were only observed in the second half of the nestmate provisioning period, suggesting an underlying change in how bees behaved towards one another. It is possible that once *X. virginica* females established dominance hierarchies within nests, individuals recognized which female would be the forager and which females would have to be fed. In a previous study, females that foraged during the nestmate provisioning phase either continued foraging as the brood provisioning phase began (so they were dominants), or they disappeared altogether and were replaced by the next individual in the reproductive queue (Richards and Course 2015). These observations suggest that the foragers were

dominants and that they fed her subordinates. If so, this would provide an interesting contrast with other social bees such as *Megalopta genalis*, in which subordinate females generally feed dominant ones (Kapheim et al. 2011). Additional evidence that feeding behaviours are related to the female dominance hierarchy formation is found in the fact that male bees were never fed.

The finding that foragers were smaller on average than the bees they fed is unexpected if foragers were dominants. This finding also contrasts with patterns typically observed later in the colony cycle; during the brood provisioning phase, large females tend to be dominant (Richards 2011; Richards & Course 2015). The small size of foragers may represent a biased estimate if pollen-feeding behaviour represents an additional incentive given by dominant females to subordinates that they cannot completely control through aggression. Aggression was more frequent when foragers returned without pollen. However, pollen feeding was not observed in all nests, and might be more frequent when smaller dominants can reinforce their social status by feeding subordinates that they cannot fully dominate by aggressive interactions. The largest dominant females may be able to establish and maintain their primary position in the reproductive queue, simply by using aggression, and then could remain in their nests during the nestmate provisioning period, avoiding the risks of foraging. Thus foraging by relatively small dominants might be more likely than by large dominants.

If spring foragers are dominants that feed subordinates, then this represents another interesting contrast between carpenter bee societies and those of other social insects, in which is is more common for subordinate individuals bring food back to the nest to appease higher ranking individuals (Lin & Michener 1972; Kukuk & Crozier

1990; Stark 1992; Hoffmann & Korb 2011). Food is even used as a peace offering during interspecific interactions. The native ants *Solenopsis geminata* and *Pheidole dentata* offer food to the aggressive, invasive fire ant *Solenopsis invicta* to reduce aggressive interactions (Bhatkar & Kloft 1977). In queenless colonies of the termite *Cryptotermes secundus*, workers that feed their nestmates more frequently are more likely to become the next queens (Hoffmann & Korb 2011).

The relatively small size of spring foragers suggests that many of them were former tertiary females, the small females that remain inside nests during their first year (Richards 2011; Richards & Course 2015). Tertiary females overwinter twice, and some manage to become dominant foragers in their second spring (Richards 2011, Vickruck unp. data), so the small average size of foragers in 2012 might reflect high survival of tertiaries from the previous year. If spring foragers are indeed dominant females, then their relatively small size in spring 2012 might suggest that age, as well as size, influences dominance status, or that spring foragers are not dominant after all. Development of better observation nests is needed to solve this conundrum.

Conclusions

Eastern carpenter bees use nestmate, rather than kin recognition when interacting with conspecifics within spring nests. Behaviour was also context dependent, and females returning to the nest with pollen were engaged in fewer aggressive encounters than those that did not. In social nests, it is more important for female bees to identify which nestmates are a part of the dominance hierarchy rather than which bees are relatives. Understanding the role of cuticular hydrocarbons among nestmates and non-

nestmates, as well as related and unrelated bees would be an interesting next step to further the understanding of the mechanisms used for conspecific recognition in *X. virginica.*

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Table 3.1. Contrasting predictions for the relative frequency of cooperative versus aggressive interactions when bees are hypothesized to discriminate group versus non-group membership based on kin recognition (relatedness cues), nestmate recognition (familiarity cues) or both. When both relatedness and familiarity cues are used, then the effect of the two types could be similar (equal strength) or one could be stronger than the other. Legend: R = related interactants, UnR = unrelated, F = familiar, UnF = unfamiliar.

	Relative frequency of behaviour	
Recognition cue	**Cooperation**	**Aggression**
Familiarity	F > UnF	F < UnF
Relatedness	R > UnR	R < UnR
Both		
Relatedness stronger	R,F > R,UnF > UnR,F > UnR,UnF	R,F < R,UnF < UnR,F < UnR,UnF
Familiarity stronger	R,F > UnR,F > R,UnF > UnR,UnF	R,F < R,UnF < UnR,F < UnR,UnF
Cues equal strength	R,F > R,UnF = UnR,F > UnR,UnF	R,F < R,UnF = UnR,F < UnR,UnF

Table 3.2. Ethogram of all behaviours recorded from observation nests of *X. virginica*. All behaviours were counted as events (E), although two behaviours (marked S) sometimes occurred for long enough to be measured as state variables. The frequency of each behaviour was calculated relative to a total of 667 behavioural events from the perspective of the focal bee. The relative number of nests in which each behaviour occurred was divided by 40. (The total number of nests in the aggregation).

Behaviour by focal bee	**Definition**	**Event or state**	**Relative Frequencies in females and males**	**Relative number of nests in which behaviour occurred**
Feeding behaviours				
Trophallaxis	Second bee rotates horizontally 180^{o} to the focal female. Both bees extend their tongues. Focal female regurgitates nectar and second female consumes it.	E	5.1%, 0%	37.5%
Scrape pollen off	Focal female rubs two hind legs together to remove pollen from her legs.	E	5.5%, 0%	55%
Eat pollen	Focal bee consumes pollen from a slant at one end of the nest.	E	2.5%, 0%	30%
Receive beg	Second bee extends her front pair of legs and repeatedly 'taps' the focal female on the head or abdomen, depending on her orientation.	E	1.8%, 0.14%	20%
Eat pollen off focal female	Second bee consumes pollen directly off focal bee. This may occur while the focal bee is engaged in an interaction with a third bee.	E	1.20%, 0%	7.5%
Aggressive behaviours				
Bite	Focal bee opens mandibles and closes them forcefully on the second bee.	E	0.14%, 0%	2.5%
C- posture	Focal bee curves abdomen under her thorax while facing second bee.	E	0.28%, 0%	2.5%
Ejected	Focal bee is forcibly pushed out of the nest entrance by second bee.	E	2.85%, 0.28%	27.5%
Push	Focal and second bee face each other and attempt to drive each other backwards through contact with their heads.	E or S	3.14%, 0%	37.5%
Receive bite	Second female bites focal female.	E	0.75%, 0.28%	17.5%
Receive C-posture	Second female displays C-posture to focal female.	E	0.45%, 0%	7.5%
Receive push	Second female pushes while focal female backs up.	E	0.14%, 0%	2.5%
Other interactions				
Pass	Focal bee meets second bee. One of the two bees turns over and they pass each other venter to venter.	E	9.1%, 0.14%	57.5%
Attempted pass	Second bee blocks focal bee from passing by pushing head and abdomen to fully block tunnel.	E	0.75%, 0%	10%
Head to head touch	Focal bee touches the head of the second bee with her head and remains motionless.	E	2.25%	25%

Individual behaviours				
Back	Focal bee moves backwards without turning.	E	0.14%, 0%	2.5%
Exit	Focal bee leaves the nest of her own accord.	E	13.64%, 0.28%	82.5%
Groom	Focal bee passes her legs or antennae through her mouthparts.	E	13.49%, 3.15%	87.5%
Still	Focal bee remains motionless.	S	2.85%, 1.65%	47.5%
Turn	Focal bee changes direction.	E	25.49%, 2.10%	92.5%

Table 3.3. Sample sizes for pairs of interacting *Xylocopa virginica* females in 2012. Related and unfamiliar pairs were very uncommon. Females that had overwintered together in the nest were classified as familiar, whereas unfamiliar females had spent the winter in two different nests. Pairs were classified as related if Kingroup analysis indicated they were significantly more likely to be full sisters than unrelated pairs.

	Familiar	**Unfamiliar**	**Total**
Related	8	3	11
Unrelated	15	33	48
Total	23	36	59

Table 3.4. Summary of behavioural interactions during the first two minutes after focal females returned to the nest with or without pollen. Females returning with pollen were the subject of more feeding behaviours and fewer aggressive behaviours than those that did not (X^2=115.83, d.f.=2, P<0.00001).

Behaviour category	**With pollen**	**Without pollen**
Feeding	95 (65.5%)	7 (6.8%)
Aggressive	8 (5.5%)	49 (48.0%)
Other	42 (29.0%)	46 (45.1%)
Total interactions	145 (100%)	102 (100%)

Table 3.5. Influence of relatedness and familiarity on feeding, passing and aggressive interactions. To control for sample size effects, tests of familiarity were conducted among unrelated pairs and tests of relatedness were conducted only among familiar pairs.

	Familiarity (among unrelated pairs only)		**Relatedness (among familiar pairs only)**	
	Familiar	Unfamiliar	Related	Unrelated
Feeding	17 (50%)	8 (16%)	6 (35%)	17 (50%)
Aggressive	8 (24%)	27 (55%)	6 (35%)	8 (24%)
Other	9 (26%)	14 (29%)	5 (30%)	9 (26%)
Total	34	49	17	39
	Fisher's exact, P=0.002		Fisher's exact, P=0.64	

Table 3.6. MANOVA table comparing the effects of relatedness, familiarity, pollen rewards and body size and the interaction effect of the focal bee on behavioural interactions between females in the first two minutes following a focal female's return to the nest. The response variable was a matrix of the number of occurrences of feeding, aggressive and other behaviours between each interacting pair of bees.

	d.f.	**Wilks**	**Approx. F**	**num d.f.**	**den d.f.**	**P**
Relatedness	1	0.952	1.06	3	51	0.40
Familiarity	1	0.862	3.29	3	51	**0.03**
Pollen	1	0.665	9.85	3	51	**0.00004**
Body size	1	0.95935	0.72	3	51	0.54
Interaction with bee identity	10	0.67043	0.73	30	150	0.84
Residuals	53					

a)

b)

Figure 3.1. Eastern carpenter bees inside the nest. a) Overwintering *X. virginica* huddled together at the end of a nest tunnel. (Image courtesy of D. Skandalis) b) solitary female after provisioning brood.

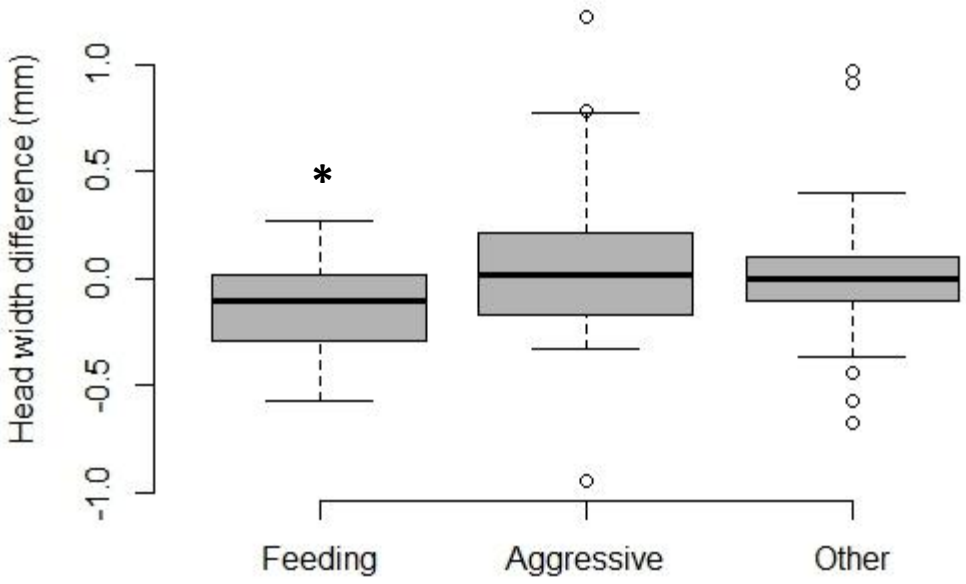
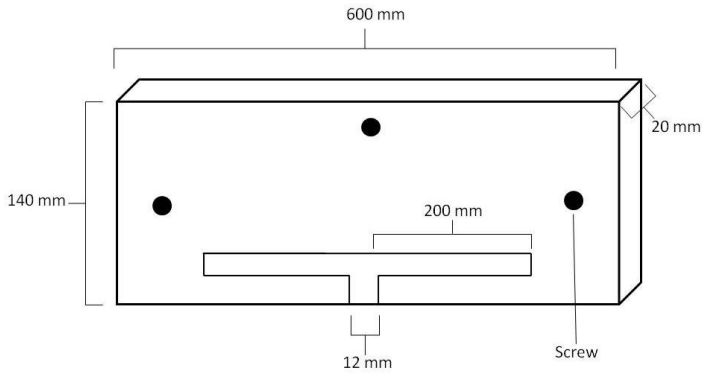


Figure 3.2. Differences in head width between females involved in feeding, aggressive, and other interactions. Head width difference was calculated as the head width of the focal bee minus the head width of the other bee. The head width of feeding bees was significantly smaller than those bees being fed (t=-3.41, d.f.=31, P=0.002). Bees involved in aggressive and other interactive behaviours were not different in size (Aggressive: t=0.891, d.f.=46, P=0.377, Other: t=-0.22, d.f.=22, P=0.823).

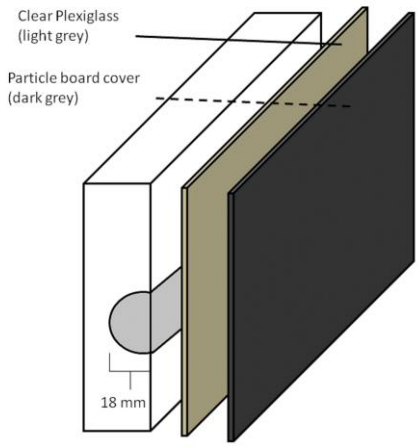
Supplementary Table S3.1. Frequency of behaviours when a female returned to the nest with or without pollen during the nestmate provisioning period. Only females brought pollen back to the nest. Beneficial behaviours with asterisks (*) were behaviours that could not have taken place if the focal bee had not brought pollen back to the nest. For all tests, degrees of freedom were equal to 1.

	Return with pollen?				
Behaviour	**Yes**	**No**	**Total**	**X^2**	**P**
Feeding behaviours					
Feed nectar (Trophallaxis)	28	6	34	14.24	**0.002**
Scrape pollen off*	37	NA	37	37.00	**-**
Eat pollen*	14	NA	17	7.12	**-**
Receive beg	11	1	12	8.33	**0.004**
Eat pollen off focal female *	5	NA	5	-	-
Aggressive behaviours					
Bite	0	1	1	-	-
C-posture	0	2	2	-	-
Ejected	4	17	21	8.05	**0.005**
Push	3	18	21	10.714	**0.001**
Receive bite	1	7	8	-	-
Receive C-posture	0	3	3	-	-
Receive push	0	1	1	-	-
Other interactions					
Pass	39	29	68	1.47	0.23
Attempted pass	2	3	5	-	-
Heat to head touch	1	14	15	11.27	**0.0008**
Individual behaviours					
Back	0	1	1	-	-
Exit	12	83	95	53.06	**<0.0001**
Groom	14	96	110	61.13	**<0.0001**
Still	2	27	29	21.55	**<0.0001**
Turn	30	156	186	85.36	**<0.0001**

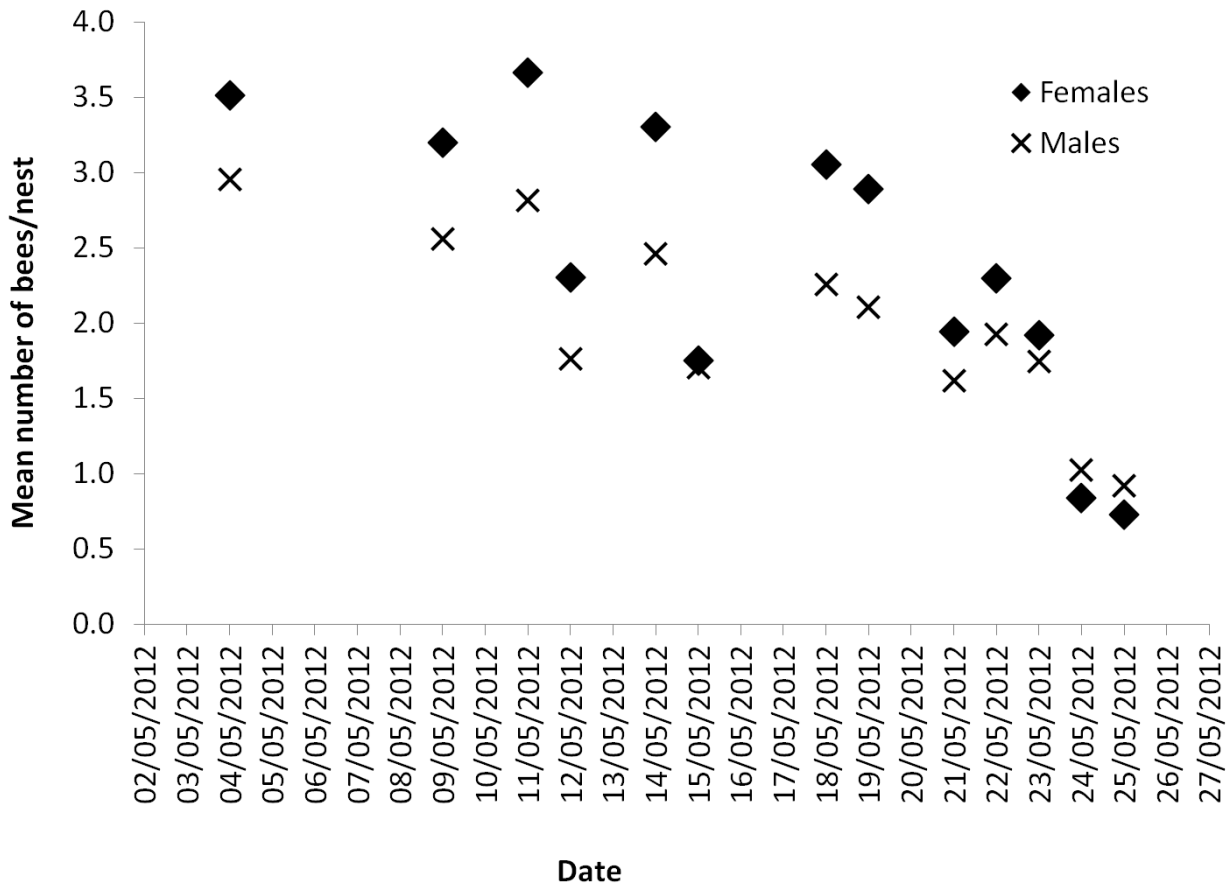
a)



b)

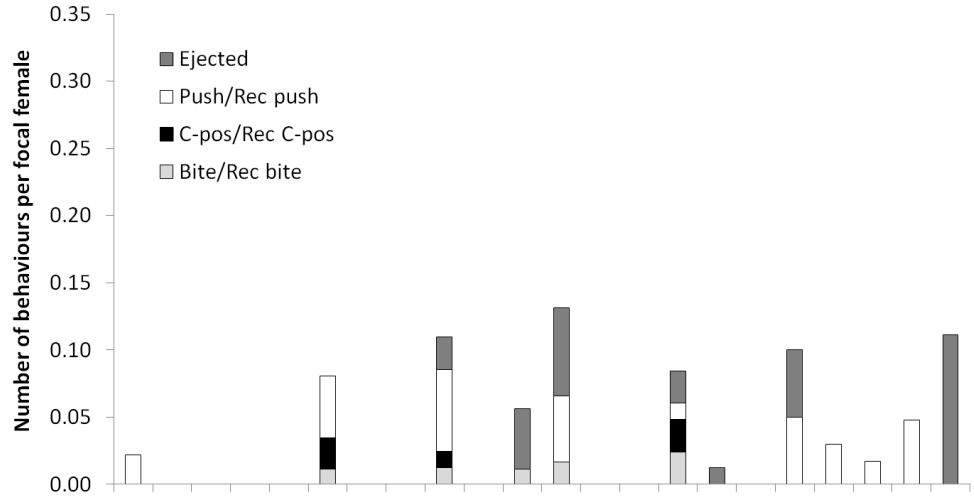


Supplementary Figure S3.1. Side (a) and end (b) view of observation nests used to house *X. virginica.* Nests were constructed from pine, particle board and plexiglass.

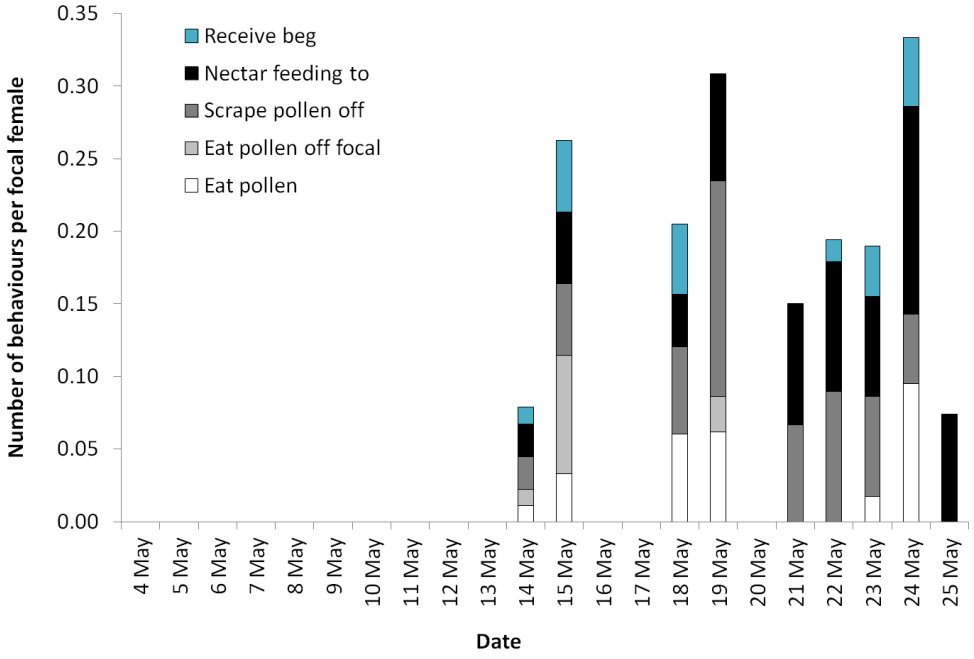


Supplemental Figure S3.2. Nest density as it changed across the nestmate provisioning period in 2012. Points represent the average number of bees in each nest on the mornings prior to observation.

a)

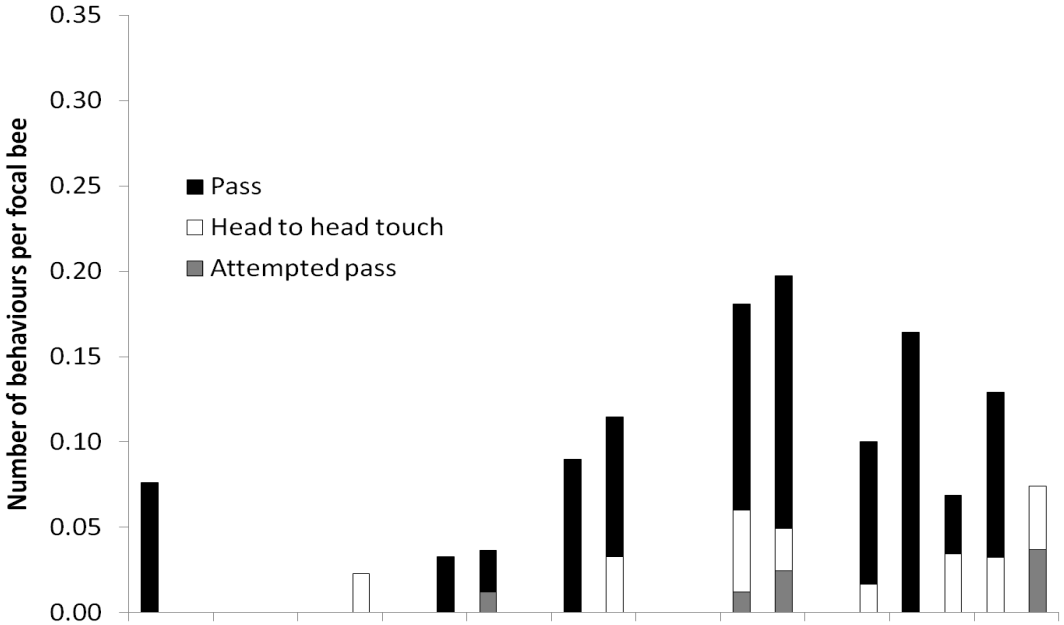


b)

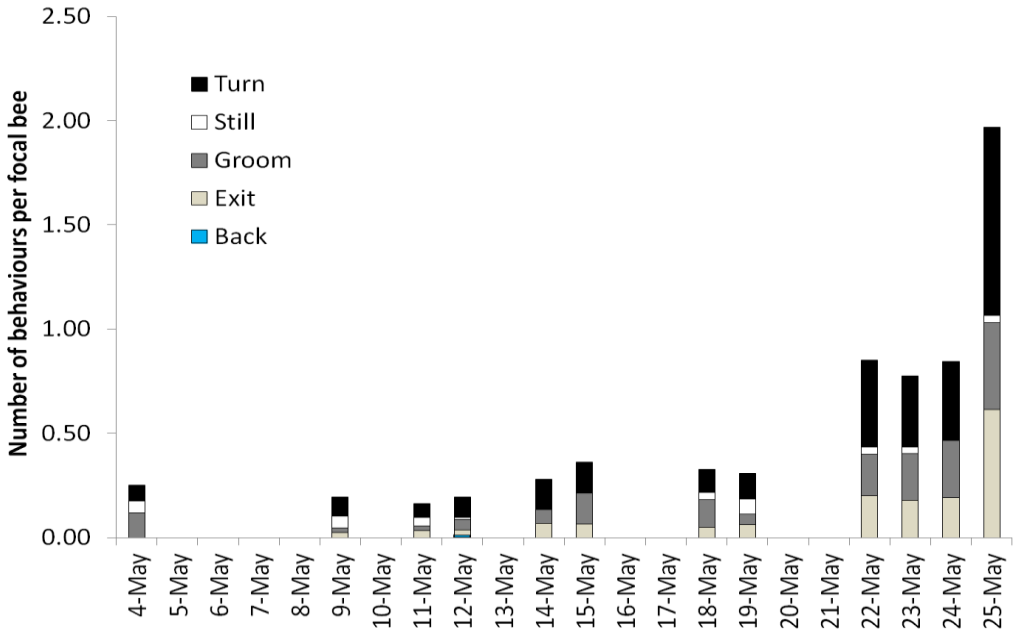


Supplemental Figure S3.3. Average number of aggressive (a) and feeding (b) behaviours per focal female per two minute encounter over the course of the nestmate provisioning phase in 2012. Per bee values were calculated based on the population size each morning.

a)



b)



Supplemental Figure S3.4. Average number of other interactive (a) and individual (b) behaviours per focal female per two minute encounter over the course of the nestmate provisioning phase in 2012. Per bee values were calculated based on the population size that morning. Note the y axes are not scaled equally.

Rationale for chapter four

Chapter 3 provided evidence that eastern carpenter bees use primarily nestmate recognition when interacting with other females inside spring observation nests. Thus, we know how female carpenter bees recognize one another, but not the consequences of this recognition when offspring are being provisioned. In the summer when eggs are being laid, not all females have equal reproductive opportunities within the nest, and a single female typically monopolizes egg laying at any given time (Richards & Course 2015). Previous observational evidence suggested that a linear queue may be present in social *X. virginica* nests, as replacement foragers were seen when primary foragers disappeared (Richards & Course 2015). This chapter aimed to explore the nature and flexibility of the reproductive queue seen in eastern carpenter bee nests with the goal of understanding why and how *X. virginica* has evolved three different types of female (primary, secondary and tertiary) within social nests. I also aimed to understand physical differences of bees in the reproductive queue and how these differences may contribute to the behaviour displayed by females when artificially moved to the front of the queue.

Chapter 4: Dominance hierarchies and conditional reproductive strategies in the facultatively social bee *Xylocopa virginica*

J. L. Vickruck & M. H. Richards

Author contributions: JLV and MHR designed the experiment. JLV collected observational data and genotyped specimens. JLV and MHR analyzed the data. MHR provided equipment and reagents. JLV wrote and MHR edited the manuscript.

Introduction

In many social groups not all individuals have the opportunity to reproduce, at least not at the same time (Michener 1974; Smith et al. 2009; Lucas et al. 2011). Competition for limited reproductive opportunities can lead to the formation of different reproductive strategies within species. This, in turn can lead to the formation of dominance hierarchies within the group, where the individual at the top of the hierarchy is reproductive while others wait for potential opportunities in the future (Dugatkin 1997; Reeve & Keller 2001). This hierarchy can be interpreted as a queue, where the reproductive individual is in queue position one. A second possible outcome is the evolution of different reproductive strategies within a species (Gross 1991, 1996; Lank et al. 1995). These strategies can involve differences in behaviour (and sometimes phenotype) among group members, and are mediated by heritable genetic traits or behavioural flexibility (Gross 1996).

Types of dominance hierarchies

In groups which form dominance hierarchies, individuals who are not reproductive can form a linear queue or remain as a non-linear grouping. A linear hierarchy suggests that individuals within the group establish a sequential dominance order, where each member has a rank which ranges between 1 (top of the hierarchy) and N (the size of the group). In linear hierarchies, group members behave in a predictable manner. Removal of the individual at position one leads to reproductive opportunities for the individual at queue position 2, and so on (Bridge & Field 2007). Linear hierarchies

are often seen in smaller social groups including species in the wasp genera *Polistes* and *Liostenogaster* (Cronin & Field 2007; Bridge & Field 2007; Ishikawa et al. 2010).

An alternate concept is that of a non-linear hierarchy. In this type of group there is no predictable pattern of nest inheritance and individuals other than the current dominant female in the nest cannot be assigned to a position in the queue. Removal of the dominant female in non-linear societies often leads to disorganization of the group, either where multiple individuals attempt to reproduce (Robinson et al. 1990), no one reproduces, or a the remaining group members rear an individual to become the new reproductive (Tarpy et al. 2000).

Alternate reproductive strategies

Two main types of reproductive strategy have been noted within species. True alternate reproductive strategies are characterized by heritable genetic polymorphisms and each strategy must have equal mean fitness over time (Gross 1996). Alternate reproductive strategies in nature are relatively rare, but have been observed in several species including the marine isopod, *Paracerceis sculptam*, and the blue gill sunfish, *Lepomis macrochirus* (Shuster 1989; Gross 1991). In contrast, conditional reproductive strategies are not genetically determined, but are the result of behavioural decisions made by individuals, and must increase the fitness of the individual in the given situation (Gross 1996). This means that the phenotype or behaviour of the individual employing the conditional strategy may vary based on individual circumstance (Gross 1996).

Factors affecting reproductive strategy and queue position

There are several factors that can affect both an individual's position within the queue or which conditional reproductive strategy they adopt, including size, age, residency status and chemical signals (Hogendoorn & Velthuis 1999; Cant et al. 2006; Bridge & Field 2007; Zanette & Field 2009; Lucas et al. 2011). Larger individuals are often better able to compete for reproductive opportunities, especially when aggressive interactions determine dominance status among group members (Hogendoorn & Velthuis 1999; Pabalan et al. 2000). However, there are several cases where body size does not influence dominance rank (Zanette & Field 2009; Smith et al. 2009; Leadbeater et al. 2011). The age of group members is also negatively correlated with dominance ranks in many species, with older individuals at the front of the queue (Hogendoorn & Velthuis 1999; Joyce & Schwarz 2007; Bridge & Field 2007). Occasionally the interplay between colony cycle and age can influence rank. Older wasps of the species *Polistes japonicus* were dominant at the beginning of the colony cycle, while younger wasps were more likely to be dominant near the end (Ishikawa et al. 2010). In addition to size and age, other indicators of reproductive quality can influence dominance status among group members. In the wasp *Polistes exclamans,* facial patterns as well as the body size were significant predictors of aggressive behaviour towards conspecifics (Tibbetts & Sheehan 2011). In an elegant study of the wasp, *Polistes dominulus,* the size of black facial markings was a significant determinant of rank while body size and age were not (Zanette & Field 2009).

Dominance hierarchies and reproductive strategies in the genus Xylocopa

Carpenter bees from the genus *Xylocopa* offer an excellent opportunity to explore how dominance hierarchies function and what physical characteristics may lead to different positions in the reproductive queue. The size of carpenter bee social groups is relatively small and division of labour is complete, with dominant individuals both foraging and laying eggs while subordinate females wait in the queue for reproductive opportunities (van der Blom & Velthuis 1988; Gerling et al. 1989; Stark 1992; Richards 2011). In *Xylocopa pubescens,* social nests usually comprise two females and the breeding season allows for two rounds of offspring provisioning per year, making for a very simple queue (Gerling et al. 1981; Hogendoorn & Leys 1993; Hogendoorn & Velthuis 1993). One female is the dominant egg layer while the second female is a subordinate guard (Velthuis & Gerling 1983). In this species, subordinate females are typically newly emerged daughters with dominant mothers, or old superseded mothers with dominant daughters, although same generation sister groups infrequently occur (Hogendoorn & Leys 1993). In *X. pubescens,* both body size and age have been shown to predict the winner of aggressive contests (Hogendoorn & Velthuis 1999). Hierarchies are similar in the multivoltine (multiple rounds of reproduction per year) *X. sulcatipes,* where two bee social nests can be mother-daughter pairs or sister pairs (Stark et al. 1990; Stark 1992). In both species supersedure by new dominant females is often accompanied by the destruction of the brood laid by the previous dominant female (Stark et al. 1990; Hogendoorn & Leys 1993). In most cases, some brood are saved and has been modeled as a staying incentive for the superseded female to remain at the nest (Dunn & Richards 2003).

The eastern carpenter bee, *X. virginica*, is a facultatively social species which nests in small social groups that are slightly larger than those of either *X. pubescens* or *X. sulcatipes* (Richards & Course 2015). Bees overwinter as adults, and the majority of dispersal takes place in spring (Peso & Richards 2010b). Females have two distinct foraging phases: the nestmate provisioning phase (NPP), where pollen is collected to feed other adult conspecifics in the nest, and the brood provisioning phase (BPP), where pollen is gathered to feed developing offspring (Richards & Course 2015; Chapter 3). The majority of dispersal happens during the nestmate provisioning phase. Bees have the option to disperse away from the local population, excavate a new nest of their own, or join a nest that had been previously established in the population. While many females disperse, others remain in their natal nest. The nestmate provisioning phase lasts 2-3 weeks and is followed by the brood provisioning phase. During the brood provisioning phase, pollen and nectar are collected and brought back to the nest to provision offspring rather than feed other adults in the nest. The brood provisioning phase typically lasts approximately 6-7 weeks.

Social *X. virginica* nests share the same complete reproductive skew seen in other carpenter bee species. There is typically a single reproductive female in the nest along with other subordinate, non-reproductive females (Gerling & Hermann 1978). *Xylocopa virginica* social groups can be larger than two females and three different types of females have been described based on wing and mandibular wear (Richards 2011). Primary females have high mandibular and wing wear, secondaries had intermediate wear scores while tertiary females have pristine wings and mandibles even at the end of the foraging season (Richards 2011). The lack of wear seen in tertiary females indicates that

they did not do any work outside or inside the nest (Richards 2011). Tertiary females can also overwinter twice, giving them an opportunity to move up the reproductive queue in their second summer. This also suggests that tertiary females may have adopted a conditional reproductive strategy of delaying reproduction (Richards & Course 2015). Previous work has shown that in social *X. virginica* nests only one female forages at a time, suggesting a linear reproductive hierarchy within nests (Richards & Course 2015). It is currently not known how tertiary females react to being at the front of the reproductive queue.

Objectives

This study aimed to link dominance hierarchy behaviour with the different reproductive strategies employed by female eastern carpenter bees. The first aim of this study was to understand the behavioural flexibility of secondary and tertiary females seen in social nests. Specifically, when presented with the opportunity to become a replacement primary, how do secondary and tertiary females respond? We used serial removal experiments across three years to determine the reproductive flexibility of both secondary and tertiary females. The second main goal was to describe the physical characteristics of primary, secondary and tertiary females, and to identify possible physical traits that may explain the reproductive behaviours employed by each female. Lastly, we aimed to track tertiary females across both years of their lives to quantify overwintering success and reproductive potential in their second year. Quantifying the behaviour of tertiary females across their lifespan will help explain why this strategy has evolved and how it has persisted in *X. virginica* social groups.

Methods

Field site and activity periods

Xylocopa virginica nests were studied at the Glenridge Quarry Naturalization Site (GQNS), in St. Catharines, Ontario, Canada (43.122, -79.236 decimal degrees). Within the park were five wooden bridges constructed over dry ditches; and each bridge housed a separate nesting aggregation of 10-22 nests (bridges named A, B, C, D and F). Eastern carpenter bees reuse nests for many years and these bridges became available to the bees in 2004, seven years prior the start of the study. All aggregations were within 500 m of one another.

In 2011 bees were first seen foraging on 12 May, and the last day of foraging was 7 July (Supplemental Figure 4.S1). That year, marking and observation of females took place after the brood provisioning phase had already commenced. In 2012, the first foraging day (also the first day of the nestmate provisioning phase) was 15 May. The brood provisioning phase began on 28 May and the lasted until 29 June (the last foraging day). In 2013, the nestmate provisioning phase began 20 May, the brood provisioning phase began 29 May and the last day of foraging was 1 July (Supplemental Figure 4.S1).

Bee capture and marking

Bees were caught at the nest by placing 'cup traps' over nest entrances. A plastic cup with a small hole in the bottom and a sealed lid was placed over each nest entrance. Bees leaving the nest became trapped in the cup, at which time they were chilled for approximately 10 min before marking and measuring.

Each bee was marked on its thorax with a unique two paint combination with Testors enamel. Head width was measured with digital calipers. Measurements in all years were done by a single person (JLV) to eliminate any measuring bias. Head width was measured across the widest portion of the head including the compound eyes. Wing wear was scored from 0–5, where 0 indicated perfect wing margins with no nicks or tears and 5 indicated wings with completely obliterated margins. After marking each bee was placed back just outside its nest entrance to resume normal activities.

Foraging observations and determination of nest status

Eastern carpenter bee nests were observed during both the nestmate and brood provisioning phases in 2012 and 2013, and during the brood provisioning phase only in 2011 to track individual female activity across the year. Once females began to fly nests were observed daily from 8:00 - 16:00 h, weather permitting. Observations ceased when an entire observation day passed (8h) without seeing a single pollen trip by a female bee, indicating that the brood provisioning phase was complete.

Cup traps were placed over all nest entrances in the morning to trap any females leaving the nest. Time and nest of departure as well as the bee's individual paint ID were recorded for each bee leaving the nest. Bees were then released and the trap was replaced over the nest entrance. When a bee returned to the nest, the trap was removed to allow her entry. The time of her return, the nest which she returned to, as well as whether or not she was carrying pollen was recorded. At the end of the day all cup traps were removed for the night.

To determine if there were bees left in the nest after a female had departed a small plastic transfer pipette was inserted into the nest entrance. Females remaining in the nest would buzz or bite the end of the plastic transfer pipette, or block the entrance with their abdomen, preventing the transfer pipette from entering the nest. The presence or absence of a guarding female was recorded and used to determine if the nest was social or solitary. Nests were classified as solitary if during the brood provisioning phase only one female was ever seen bringing pollen to the nest and a second bee was never seen guarding the nest entrance. Nests were classified as social if more than one female was recorded in the nest during the brood provisioning phase.

Assigning reproductive strategies to female bees

Assigning reproductive strategies to *X. virginica* females can be challenging, as it is difficult to know which females are inside a nest at any given time without destroying it. Richards (2011) categorized females at the end of the foraging period based on wing and mandibular wear patterns. In this study, we categorized females based on flight and foraging activity, as it was necessary to categorize primary females during the reproductive period in order to conduct removals. Females were categorized as primaries if they made at least three pollen trips over an eight hour observation period. This is approximately the number of mean pollen trips per bee per day during the brood provisioning period as reported by Richards and Course (2015). Rarely, two females made three or more pollen trips on the same day. Under these circumstances both females were designated primary foragers. Following the removal of a primary female, a new bee

was categorized as the replacement primary if she made at least three pollen trips over an 8 hour observation period.

Secondary and tertiary females were more difficult to categorize on the wing. As such, they were classified at the end of the brood provisioning phase based on flight and foraging activity across the foraging period. Bees were categorized as secondary females if they were seen flying outside of the nest more than once during the brood provisioning phase. Secondary females could make pollen trips, but never more than two in a single observation day.

Tertiary females were defined as bees that were never seen flying outside the nest. Thus, in order to identify tertiary females all other bees in the nest had to be removed, after which these females have to eventually leave the nest to feed. Tertiary females had to be previously unmarked and unworn, reinforcing that they had likely not left the nest. Tertiary females were permitted to have wing wear scores of one, as occasionally a small amounts of damage can occur inside the nest.

Forager removals and dissections

Nests at each of the five bridges were assigned to one of three groups: solitary, control or social removal (Supplemental Figure 4.S2). No nests thought to be solitary at the beginning of the brood provisioning phase became social by the end of the year. Nests in the control category were social and contained more than one adult female during the brood provisioning phase. Females in control nests were marked and measured in the same manner as females in experimental nests. Control nests were used to quantify female foraging activity and to observe the rate at which primary foragers are

replaced in unmanipulated nests. Social removal nests also contained more than one adult female and were subjected to removal experiments to understand the nature of the reproductive queue in each nest.

Once selected for removal, primary females were caught in cup traps when leaving the nest and immediately stored in 70 or 100% ethanol on ice in the field. Removed females were transferred to a -20°C freezer at the end of each day. Removal females were dissected in the lab to assess ovarian development, matedness, and abdominal fat body content. Females were categorized as fat if they had any visible fat deposits in their abdomens and skinny if there were no visible fat deposits. We used nests in which all females had been removed or marked to accurately estimate the number of females per nest during the brood provisioning phase. To examine how the timing of removal may influence the behaviour of other females in the reproductive queue different removal regimes took place across years. Details of removals are described in detail below.

2011 Removals

In the first year of data collection, removals took place in eight of seventeen nests at a single *X. virginica* nesting aggregation (Bridge F). Of the nine other nests in the aggregation, eight were used for a separate experiment and one was a solitary nest. There were no control nests in 2011. Primary females were removed from eight nests on 13 June 2011. Daily observations continued at Bridge F until foraging activity had ceased at all nests (7 July). During this time, any female from a removal nest that made three or more pollen trips on a single day was deemed the replacement primary and was removed

via cup trap the next time she left the nest. The final bee in removal nests was marked and measured if captured, but returned to the nest and allowed to overwinter to assess overwintering survival.

2012 Removals

Of 69 social nests across all five nesting aggregations (A, B, C, D and F), 22 were designated as control nests, 38 were designated as removal nests, and 9 were new nests constructed that spring (Supplemental Figure 4.S2). Females from two newly constructed nests were also removed. Fifty-seven females were removed from the 40 social removal nests.

To test if the timing of removal of a primary forager would affect the likelihood that a replacement primary would take over, we implemented two removal points, early and late. Nests in the early removal category had their primary females removed 30 May– 6 June 2012. Nests in the late removal category had primary females removed 13 June– 15 June 2012. The last female to emerge from the nest was marked and measured when possible but never removed and allowed to overwinter.

2013 Removals

Twenty-eight females were removed from 17 nests in five nesting aggregations during the brood provisioning phase of 2013. The number of removal nests in 2013 was lower due to the increased frequency of solitary nesting in the population. (Differences in the proportion of solitary and social nests between years will be addressed in Chapter 5). There was only one removal period in 2012 which began on 9 June. Replacement

primary and tertiary females were removed from experimental nests so that ovarian development, fat content and matedness could be assessed in tertiary females as well (Supplemental Figure 4.S2).

Data analysis

All data analyses were conducted in R version 3.1.2, running in RStudio version 0.98.1056 (RStudio, Boston, Massachusettes, USA). Nests from bridge F were used in all three years of the study, while nests from bridges A, B, C, and D were used in 2012 and 2013. While nests were used across multiple years of study, we consider them independent units as it is the individuals inside the nest as opposed to the physical nest structure itself that were the subject of the analyses. Several variables including wing wear, ovarian development, rate of wing wear accumulation, and the number of bees per nest were not normally distributed and were analyzed using Kruskal-Wallis tests. Fisher's exact tests were used to examine if the last bee in the queue was significantly more likely to be a secondary or a tertiary female and to test if the number of nests from which immatures were ejected differed across years. Chi-square analysis was used to ask if the proportion of females with fat in their abdomens changed across reproductive strategy. Lastly, to understand the effects of body size on reproductive strategy we used a linear model with head width was the response variable. Explanatory variables nested the main effect of female reproductive strategy within nest to account for within nest variation among groups.

Results

Foraging behaviour in control nests

Control nests were used to describe the behaviour of the primary female and to quantify the frequency at which primary females were replaced across the brood provisioning phase. All nests in 2013 and most nests (21/22, 95%) in 2012 contained a primary forager as described using the criteria of three pollen trips per eight hour observation period (Table 4.1). The one nest in 2012 without an assigned primary forager did contain females who made pollen trips, just never three or more in a single observation day. It is possible that this nest did contain a primary female, as she could have made additional pollen trips outside of the eight hour observation window. One nest in 2012 and one in 2013 contained two primary females simultaneously foraging within a single nest. In 2013, four control nests were destroyed by ants from the genus *Crematogaster* during the brood provisioning phase.

In twenty-three percent of nests in 2012 and fourteen percent of nests in 2013 the primary female was succeeded by a second forager mid-season (Table 4.1). In 2012 the last observed foraging day was 29 June, but in three nests foraging finished on or before 13 June, more than two weeks before the end of the brood provisioning phase. A female was present inside all three nests, but never foraged. These three nests may also represent instances where the primary female did not survive to the end of the brood provisioning phase, but the final female in the nest did not become a replacement primary.

Nest composition as estimated from removal nests

Removal nests were used to estimate group sizes and reproductive strategies, as females often remain inside the nest when they are not the primary forager. Solitary nests always contained one female who was also the only bee making pollen trips across the season. Social nests varied in size from two to four females (Table 4.2). Most nests containing two females were composed of a primary and a tertiary female (30/44 nests), while 9/44 two-bee nests contained one primary and one secondary female. All nine of these nests were newly constructed in 2012. The final five two-bee nests contained a primary female and a female that was initially identified as a tertiary female (the final female in the nest who had not been seen outside the nest until all other females had been removed). However, after a period of time these females did begin to forage, making them difficult to classify based on the criteria for primary, replacement primary, secondary or tertiary bees. These bees were placed in their own group and will be referred to as late foragers, since they initially presented as tertiaries but eventually made pollen trips, technically making them replacement primaries.

All social nests with three females contained one primary female, one secondary female and one tertiary female, and all social nests with four females contained one primary, two secondary females and a tertiary female. The number of bees per nest varied significantly among years and was highest in 2012 and lowest in 2013 (Kruskal-Wallis $X^2=37.57$, d.f.=2, $P<0.00001$, Table 4.2).

The structure of dominance hierarchies within social nests

The vast majority of social nests (109/113, experimental and control nests combined) contained a single primary forager at the start of the brood provisioning phase. Only four nests across all three sample years contained two primary females that foraged simultaneously. Two of these were control nests (one nest in 2012 and one nest in 2013) and two were removal nests (both in 2012). All removals with the exception of one (87/88) had one of two outcomes: either a single female began to forage and became the replacement primary in the nest, or the nest became inactive, with a tertiary female remaining inside, leaving the nest very occasionally for nectar feeding but never making a pollen trip. In no removal nest did a female retain secondary status (making less than two pollen trips a day) once the primary female had been removed, meaning that all replacement primaries were initially secondary females. In a single nest in 2012, the removal of the primary female resulted in two secondary females simultaneously becoming replacement primaries in the nest.

The final bee in the queue was significantly more likely to be a tertiary female than a secondary female (Fisher's exact $P<0.00001$). In nests where the removal of the primary left two or more females in the nest, a secondary female always began to forage and became the replacement primary (31/31 removals). After 33 removals only one bee remained in the nest. In 28/33 occurrences the final bee remained in the nest as a tertiary female, never making a pollen trip. After 5/33 removals the final bee in the nest eventually became a late forager. These five females all belonged to two-bee nests.

Following the removal of the primary bee, females in the nest behaved differently with respect to foraging (Kruskal-Wallis $X^2=33.19$, d.f.=2, $P<0.0001$; Figure 4.1).

Secondaries who became replacement primaries made their first pollen trip on average 5.04 ± 3.17 (range 1-13) days after the primary female was removed and late foragers took 8.5±3.8 (range 4-13) days. Tertiary females did not make pollen trips, but this was not because there was no time remaining in the brood provisioning phase. On average, 15.95± 4.9 (range 7- 31) days remained until the end of the foraging season.

Physical characteristics of primary, secondary and tertiary females

Head widths of females in social nests varied across female type, but also within nest. A linear model that investigated the difference among head widths of primary, secondary, tertiary and late foraging females nested the main effect of reproductive strategy within nest, accounting for size differences among individuals within each nest. This model found reproductive strategy on its own was significant, with late foraging females being smaller than primary, secondary or tertiary groups ($F=7.27$, d.f.=3, $P=0.01$). The interaction between reproductive strategy and nest was also significant, indicating that head width differences within nests were important (reproductive strategy nested within nest $F=2.99$, d.f.=164, $P=0.048$, overall model $F_{(8,166)}= 3.07$, $P=0.04$). Primary females were on average 0.14 ±0.39 mm (1.8%) larger than tertiaries and 0.05 ±0.34 mm (0.6%) larger than secondaries in their own nests. Secondary females were on average 0.19 ±0.36 mm (2.6%) larger than tertiaries with which they were nesting.

Patterns of wing wear differed among different primary, secondary and tertiary females (Figure 4.4). At the time of removal, primary females had the most worn wings, followed by secondary females and then tertiary females (Kruskal-Wallis $X^2=35.80$, d.f.=2, $P<0.00001$; Figure 4.4a). However, primary and secondary females accumulated

wing wear at similar rates (Kruskal-Wallis X^2=0.85, d.f.=1, P=0.36; Figure 4.4b). Tertiary females were removed at first sight and were not given the opportunity to accumulate wing wear.

Dissections of females removed from social nests showed differences in ovarian development and fat stores among different types of females. Ovarian development was significantly higher in primary and secondary females than in tertiary bees (Kruskal-Wallis X^2=8.47, d.f=2, P=0.01; Figure 4.4). Females of different reproductive strategies also showed differences in whether or not fat was present in their abdomens. The majority of primary females contained no fat in their abdomens, while all tertiary females dissected had abdomens that contained fat stores (X^2=38.33, d.f.=2, P<0.00001; Table 4.3). Of the 88 females dissected, all were mated with the exception of 1 tertiary female from 2012.

Longevity and success of tertiary females

Over the three years of the study, we were able to track the fate of tertiary females that had been marked from the previous year. Ten tertiary females were marked at a single aggregation during the 2011 field season. Seven (70%) of them successfully overwintered twice and were seen again in 2012 . Two of these seven bees (29%) became primary females in their second summer, 2 (29%) became secondary females and 3 (42%) were never seen again. In 2012, fourteen tertiaries were marked across all aggregations, six (43%) of which were seen again in 2013. Three (50%) became solitary females in 2013 and three were not seen again. On three occasions females that were

primaries or secondaries were observed in the spring after their second winter, but none were seen in the aggregation during the nestmate provisioning phase.

Tertiary females were also seen ejecting immature offspring of primary females and replacement primary females out of experimental nests. This behaviour only occurred in experimental nests after all primary and secondary females had been removed. Offspring ejection was observed in 2/8 (25%) of removal nests in 2011, 8/40 (20%) removal nests in 2012 and 4/17 (24%) removal nests in 2013. The proportion of removal nests from which immatures were ejected did not differ across years (Fisher's exact, P=0.87). In contrast, offspring were never ejected from control nests.

Discussion

Dominance hierarchies and conditional reproductive strategies in social nests

Behavioural observations and removal experiments suggest that social eastern carpenter bee nests can display both linear dominance hierarchies as well as conditional reproductive strategies simultaneously in the same nest. When a primary female was removed from the nest and a secondary female remained, she always assumed the role of replacement primary. This indicates that primary and secondary females form linear dominance hierarchies; primary females are dominant to secondary females in social nests. Both types of female are ready to capitalize on reproductive opportunities that may be presented to them, and along with solitary females have assumed the conditional strategy of breeding in the current reproductive year. Turnover of primary females occurs in nests (control nests in this study, Richards and Course 2015), and the ability of secondary females to become replacement primaries allows them to capitalize on direct

fitness opportunities as they become available. Linear dominance hierarchies are also seen in other species of bee and wasp such as primitively eusocial wasps from the genus *Liostenogaster* and *Polistes* (Bridge & Field 2007; Zanette & Field 2009; Ishikawa et al. 2010). Simple two-bee dominant-subordinate relationships have also been documented in *Xylocopa pubescens* (Hogendoorn & Leys 1993), suggesting that linear dominance hierarchies have evolved in several species to combat conflict over reproductive opportunities.

In contrast, tertiary females appear to have removed themselves from the reproductive queue in an attempt to delay reproduction an entire year. By doing so, these females have adopted a conditional reproductive strategy and have evolved the ability to double their lifespan. It also appears that after the decision to delay reproduction has been made it is largely inflexible. Removal experiments demonstrated that this decision must be made during the nestmate provisioning phase, since the vast majority (28/33) of females who were initially categorized as tertiaries did not forage in the brood provisioning phase even when all other bees had been removed from the nest.

Conditional reproductive strategies in social nests are far less common in bees. There are however, several desert specialist bees that can delay emergence until enough rain has fallen to induce flowering of their host plant (Rozen Jr. 1990; Houston 1991; Danforth 1999). Some, such as *Amegilla dawsoni,* for as many as 10 years (Houston 1991). However these species are in diapause, and while tertiary females do not forage or lay eggs, they are still capable of flight and nest defence when necessary.

While most of the final bees in the nest did not waiver from their tertiary status, five females did begin to forage after an extended period of time. Their eventual foraging

activity suggests they should be classified as secondary females, but their position in the queue (last) and the especially long delay to the onset of foraging suggest that perhaps they were tertiary females who were able to alter their reproductive strategy mid-season. These five females were also among the smallest bees from the dataset, a physical characteristic associated with tertiary females (discussed in further detail below). This group, termed here as 'late foragers' adds additional complexity to the system, and also further demonstrates the remarkable flexibility of this bee.

Differences among different types of bee and their behaviour in the queue

Previously, different types of females were characterised at the end of the reproductive year based on wear and ovarian development (Richards 2011). Here, we find that behavioural observations can also be used to classify different types of females. Below, I also describe physical characteristics of each type of female, focussing on tertiary females for which we know the least.

Primary

Primary females in social carpenter bee nests are active reproductives and on average are larger than tertiary, but not secondary, females. The vast majority of nests contain only one primary forager, indicating that there is typically only one position at the top of the reproductive queue. Primary females also have high ovarian development, worn wings and minimal fat stores in their abdomens.

Because they are in queue position one, primary females immediately have direct fitness, provisioning offspring of their own. In belonging to a queue, primary females

may also have assured fitness returns if they do not survive the season (Gadagkar 1990; Smith et al. 2003). In this study, primary survival was fairly high (79% in 2012 and 86% in 2013) suggesting that reproductive opportunities for secondary females may be limited. Turnover by the primary forager was much higher in a previous study of *X. virginica* (40-65% of nests, Richards & Course 2015) indicating that high levels of seasonal variation likely alter primary survival across the season in any given year.

Secondary

Secondary females are also larger than tertiaries (but not primaries) and have ovarian development comparable to that of dominant females. Some secondary females contain fat stores in their abdomens while others did not. Secondary females have intermediate levels of wing wear, although they accumulate it at a similar rates to primary females.

When given the opportunity to become the reproductive female in the nest, secondary females always began to forage and reproduce, meaning that all replacement primary females were initially secondary females. Taken together with their high ovarian development, this indicates that secondary females are reproductives waiting for opportunities to lay their own eggs, and are not secondaries for some other reason such as subfertility. The subfertility hypothesis suggests that subordinates are less fecund than dominant females in the nest, which helps explain their subordinate ranking (Craig 1983) and has been supported in the paper wasp, *Ropalidia marginata*, (Gadagkar 2016), but not in the hover wasp, *Liostenogaster flavolineata*, or the sweat bee, *Megalota genalis*,

where subordinate females appeared as fertile as dominant females in the nest (Field & Foster 1999; Smith et al. 2009).

Tertiary

Tertiary bees are both physically and behaviourally distinct from primary or secondary females. They are smaller than primary or secondary bees and have almost no wing wear. All dissected tertiary abdomens contained large fat deposits but a surprising amount of ovarian development considering they were not actively making pollen trips. When given the opportunity to become the new dominant female in the nest, tertiary females remained inactive, occasionally ejecting the offspring of the previous dominant in the nest.

Given that it appears tertiary females are attempting to delay reproduction until the following year, the amount of ovarian development detected is a bit surprising. One possibility is that the presence of primary and secondary females suppresses ovarian development in tertiary females, and once all of the females in the nest were removed ovarian development was no longer restricted. Aggressive behaviour by dominant queens was shown to suppress ovarian development in workers of the sweat bee, *Lasioglossum zephrum* (Michener & Brothers 1974). Aggressive interactions have already been observed within nests (Chapter 3), so it is possible that the removal of all other females in the nest allowed tertiary females to reactivate their ovaries.

Tertiary females also had significantly more fat in their abdomens than primary or secondary females. Fat stores are an important source of energy during diapause, and if tertiary females are attempting to overwinter twice having a sufficient fat stores would

likely increase overwintering success. Interestingly, Gerling and Hermann (1978) mention they could discern one year old and two-year-old bees by the amount of fat in their abdomen, but do not provide details of how abdominal fat stores relate to behaviour in the nest.

All but one tertiary female had mated by the time of her removal. This indicates that tertiaries were not disadvantaged in that they could only lay male eggs. Because tertiaries were never observed leaving the nest, this also indicates that mating may have taken place inside. Males are often allowed to enter different nests at the end of the day (Peso & Richards 2010b), potentially permitting mating between unrelated pairs inside. Other twig nesting carpenter bees from the same subfamily (Xylocopinae) have been observed mating inside the nest (J. Vickruck, pers. obs.). If tertiaries mate in their first year, they may have a slight advantage in the spring, as they would not have to mate prior to provisioning female offspring. It was noted by Richards and Course (2015) that one second year female began provisioning much earlier than the rest of the population.

Tertiary females in removal nests were observed occasionally ejecting the offspring of previous primary females in the nest. The killing of immature offspring produced by the previous dominant in the group has been seen in mammals (Packer & Pusey 2008), birds (Schmaltz et al. 2008; Quinn et al. 2010) and insects (van der Blom & Velthuis 1988; Hogendoorn 1996). Interestingly, in *X. virginica*, immature offspring were only ejected from the nest by tertiary females after all other bees in the nest had been removed. This suggests that tertiary females are prevented from killing larvae and pupae while primary and secondary females remain in the nest. One explanation for this behaviour is that tertiaries are removing their competition for reproductive opportunities

in the subsequent spring. By ejecting larvae and pupae that will compete with her for reproductive opportunities, tertiary females increase their chances of becoming the primary or perhaps even solitary female in the nest. Gerling and Herman (1978) noted that one of the nests they were using for X-ray analysis appeared to lose larvae at one point in the season. Destruction of immature brood cells has been seen in the carpenter bee *Xylocopa pubescens*, where usurping females often destroy much of the brood laid by the previous dominant female (Hogendoorn & Leys 1993). In most *X. pubescens* nests not all of the brood were destroyed, with some left as an incentive for the old dominant female to stay and help guard the nest (Dunn & Richards 2003). Tertiary females in these populations do not have to worry about staying incentives, as they were the only females in the nest when brood were ejected.

Conclusions

Eastern carpenter bee females in social nests display two different conditional reproductive strategies, either attempting to breed in the current year, or delaying reproduction until the following year. Primary and secondary females who are hoping to reproduce in the current year form linear dominance hierarchies, while tertiary females who are delaying reproduction rarely forage even when given the opportunity. Tertiary females are able to maintain fat stores, minimize wear and likely alter some key physiological components to double their life span. Given that reproductive hierarchy decisions are not sorted until after spring emergence, behavioural flexibility must be maintained across all individuals, as conditional reproductive strategy decisions are likely made based on interactions within the nest in spring.

Table 4.1. Characteristics of control nests from 2012 and 2013. No nests were left undisturbed in 2011. Twenty-two nests were used as controls in 2012 and eighteen nests were used as controls in 2013. In 2013, four nests were destroyed by ants from the genus *Crematogaster*.

	Sample year	
	2012 (*N* = 22)	2013 (*N* = 14)
Nests with primary forager	21/22 (95%)	14/14 (100%)
Primary forager succeeded by another bee	5/22 (23%)	2/14 (14%)
Nests with co-foraging primaries	1/22 (5%)	1/14 (7%)

Table 4.2. Population and nest sizes of *X. virginica* across sample years. a) The number of females marked and nests observed each year across all nests. (Both control and experimental). b) The distribution of different sized nests during the brood provisioning phase. Nest sizes were calculated from solitary and removal nests only, where all females inside the nest could be counted.

a)

	Year		
	2011	**2012**	**2013**
Total females marked	42	189	101
Total nests observed	17	71	64

b)

	Year		
Nest size	**2011**	**2012**	**2013**
Solitary	1 (11%)	2 (4%)	26 (59%)
2 females	7 (78%)	23 (45%)	14 (32%)
3 females	0 (0%)	22 (43%)	3 (7%)
4 females	1 (11%)	4 (8%)	1 (2%)
Nest total	9	51	44
Mean females/nest	2.11±0.78	2.55±0.70	1.52±0.73

Table 4.3. The number of females from different reproductive strategies that either possessed fat in their abdomen (fat) or did not (skinny) across all years. Primary females were the dominant foragers in the nest, secondary females were replacement foragers at the time of removal. Tertiary females were the last females to be removed from each nest.

Reproductive strategy	**Skinny**	**Fat**	**Total**
Primary	55 (93%)	4 (7%)	59
Secondary	13 (65%)	7 (35%)	20
Tertiary	0 (0%)	9 (100%)	9
Total	68	20	88

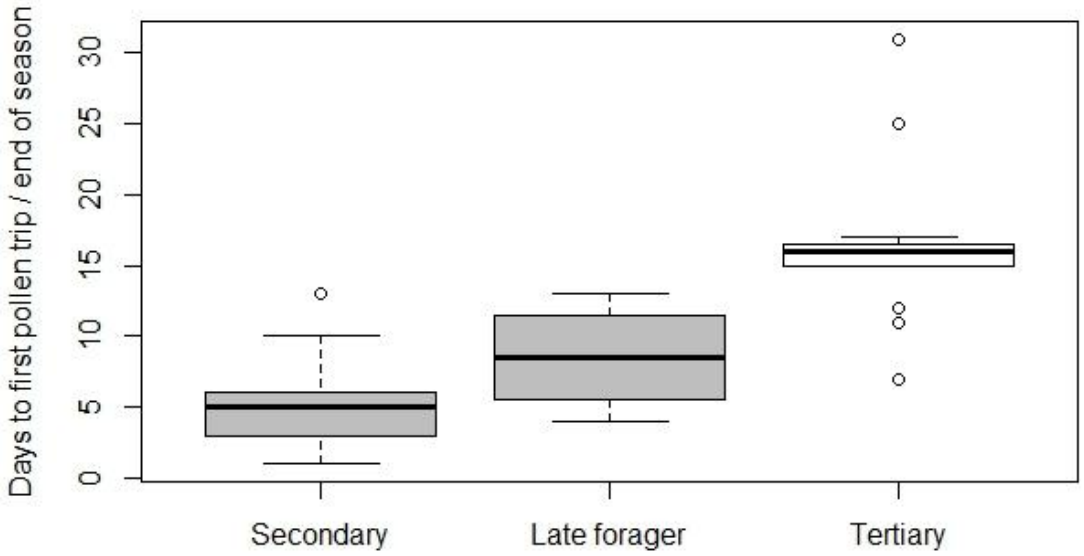


Figure 4.1. Differences in female foraging behaviour following the removal of the primary bee. Data presented for tertiary females represents the number of days until the end of the brood provisioning phase, indicating the time remaining in the brood provisioning phase which could have been used for foraging.

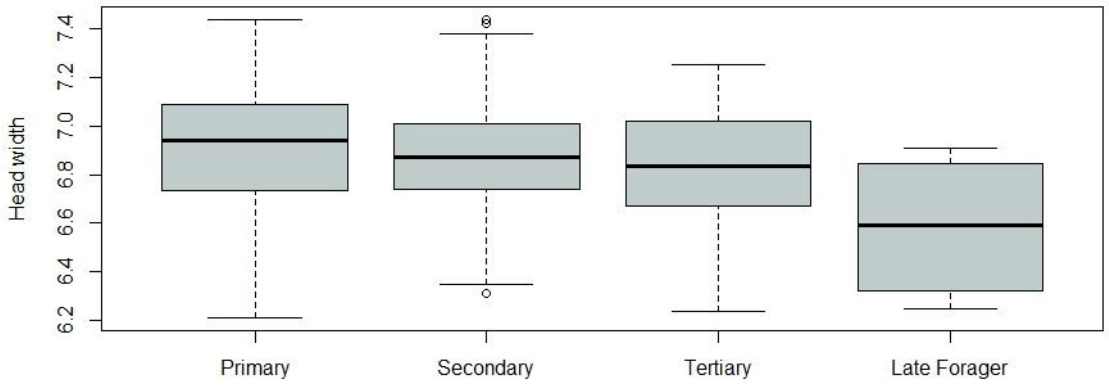


Figure 4.2. Head widths among different types of females in social nests. Data includes all females to which a strategy could be assigned from both experimental and control nests. Late foragers were bees who initially presented as tertiaries (non-foragers who did not leave the nest unless all others had been removed) but began to forage after an extended period of time.

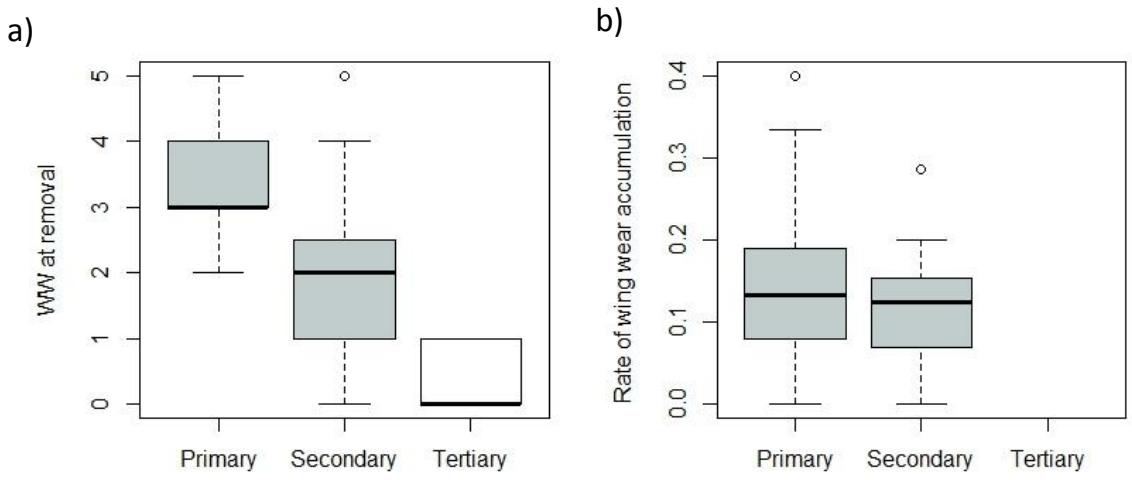


Figure 4.3. Wing wear patterns across different types of females in removal nests. a) Wing wear at time of removal. b) Rate of wing wear accumulation calculated as wing wear at time of removal minus wing wear at time of marking divided by the number of days between the two dates. All secondary females were replacement primaries at the time of removal.

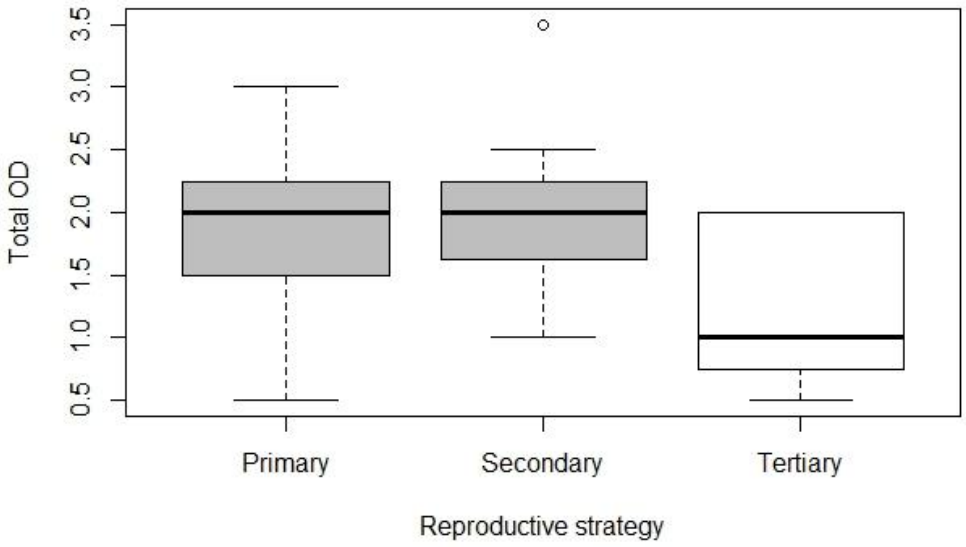
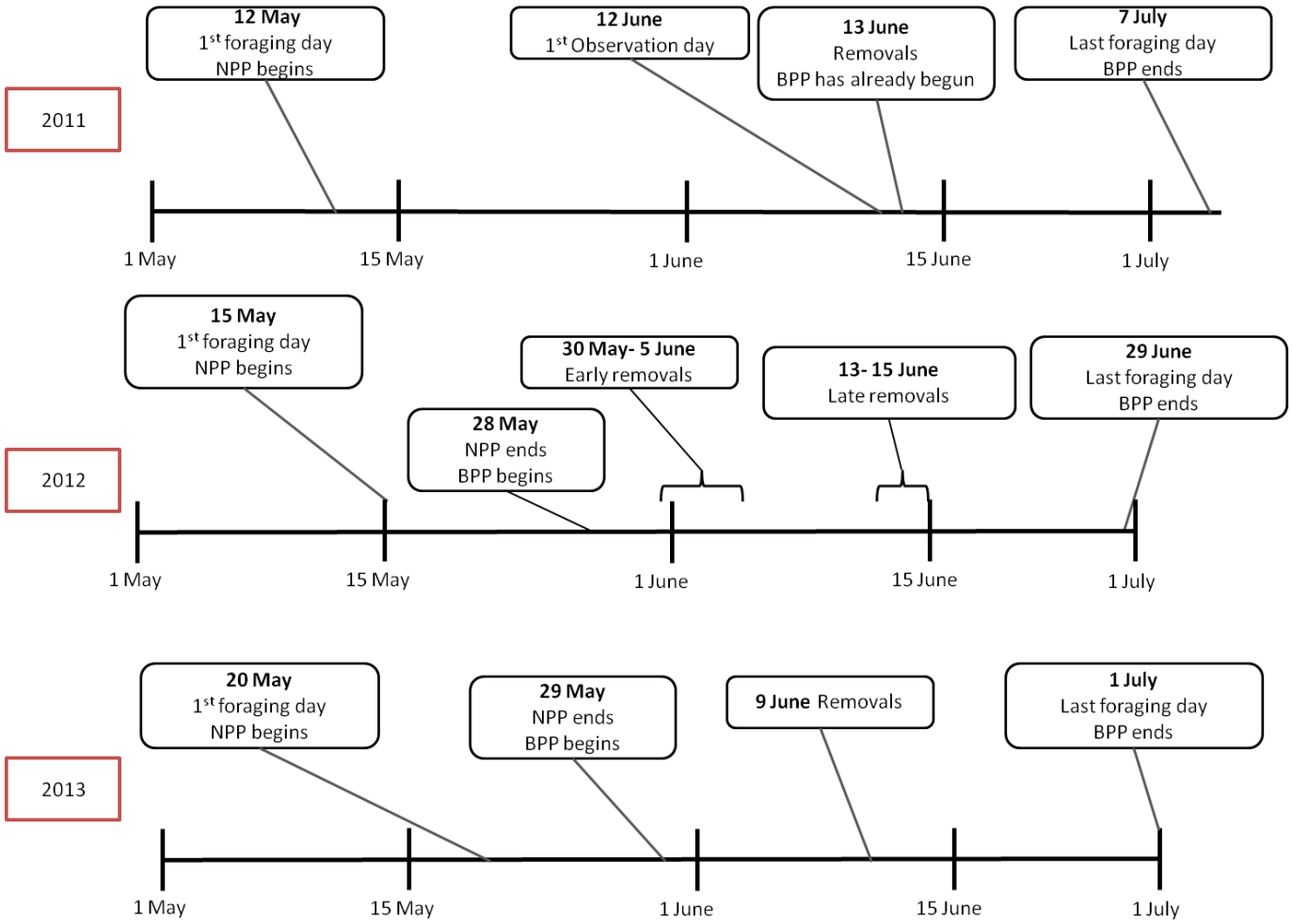
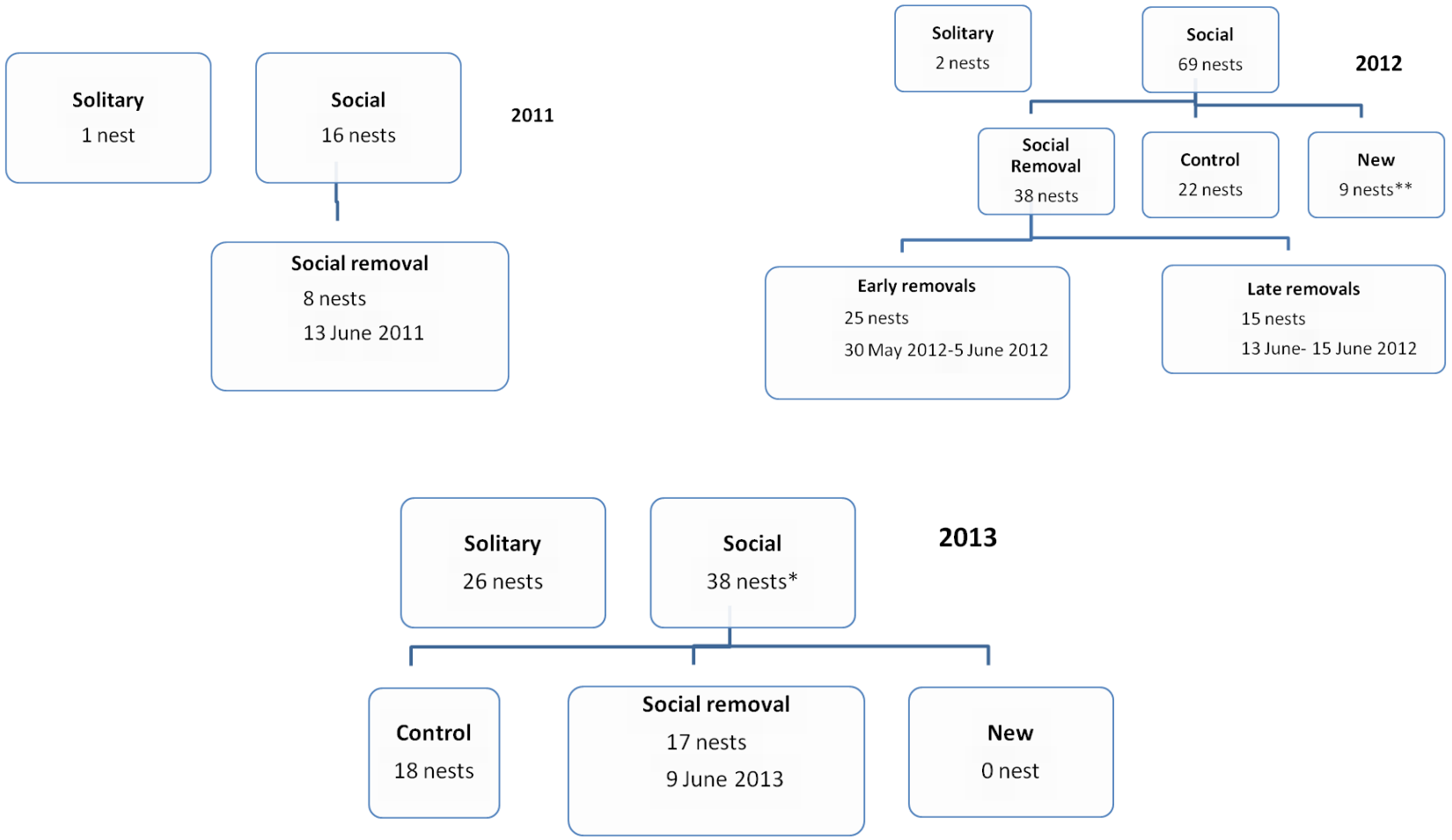


Figure 4.4. Total ovarian development for females removed from experimental nests in 2012 and 2013. All secondary females were replacement primaries at the time of removal. Grey boxes represent groups of reproductive females, white indicates non-reproductive females.



Supplemental Figure 4.S1. Time line of nest activity and removals across each year. NPP= nestmate provisioning phase, BPP= brood provisioning phase. Daily observations were made from 12 June until the last foraging day (7 July) in 2011 and from the first foraging day until the last foraging day in 2012 and 2013.



Supplemental Figure 4.S2. Flow chart of nest removals during the brood provisioning phase from 2011-13. In 2011, 9 females were removed from 8 nests. In 2012, 56 females foraging females were removed from 40 nests. In 2013, 28 bees including the last female in the nest were removed from 17 nests ** Two of the nine new nests were included as experimental nests. * Two social nests were destroyed by ants in the spring before the start of the nestmate provisioning phase.

Rationale for chapter five

Chapter four explored the formation of dominance hierarchies within social nests of eastern carpenter bees. While the previous two chapters explored the nature of social interactions among females, one of the most interesting aspects of *X. virginica* biology is its ability to nest both solitarily and socially. This flexibility in life history allows us to investigate under what conditions females nest socially or solitarily in a population. Previous studies in Niagara have reported solitary nests, but they have always occurred in very low numbers (Richards 2011; Richards & Course 2015). I encountered two very different field seasons, one in which solitary nests were common and one in which they were rare, allowing me to examine what ecological conditions promoted solitary versus social nesting in this species. In addition, while social nests are common in eastern carpenter bee populations, the genetic relationships among the females that make up these social groups was unknown. The goal of this chapter was to understand under what conditions social versus solitary nests are seen in the population, and to elucidate the forces contributing to group relatedness within social carpenter bee nests.

Chapter 5: Nest site and kin competition lead to the evolution of unrelated social groups in the eastern carpenter bee

J. L. Vickruck and M. H. Richards

Author contributions: JLV and MHR designed the experiment. JLV collected observational data and genotyped specimens. JLV and MHR analyzed the data. MHR provided equipment and reagents. JLV wrote and MHR edited the manuscript.

Introduction

How and why social groups form and are maintained has been a persistent question in behavioural ecology for decades and has been recognized as one of the major transitions in evolution (Smith & Szathmary 1995). Undoubtedly, one of the major players in this process is competition (Kokko et al. 2004; Smith et al. 2009; Van Dyken 2010). Competition can occur externally for breeding territories or nesting sites (Emlen 1982; Crespi & Ragsdale 2000; Kokko et al. 2001), as well as within the group for reproductive opportunities (Schmaltz et al. 2008; Smith et al. 2009). However, internal and external competition are not mutually exclusive, and the interplay between these two competitive forces will influence the evolutionary trajectory of individual species.

The role of resource availability in social group formation

Limited availability of critical resources can influence the amount of competition taking place among individuals looking for reproductive opportunities in the external environment. In particular, when reproductive territories are limited or costly to construct, competition for them is increased among members of the population (Hatchwell & Komdeur 2000; Schoepf & Schradin 2012). One mechanism that reduces competition for reproductive territories is to share them among a group of individuals (Hatchwell & Komdeur 2000; Kingma et al. 2014). If reducing competition through sharing territories results in an increase in overall fitness, group living can have higher reproductive success per capita than solitary living while territories are limited (Kokko et al. 2001; Kingma et al. 2014). The creation of new territories or a reduction in the population density will decrease the cost/benefit ratio of social nesting such that solitary

nesting will have higher individual fitness than social nesting. This in turn can lead to fluctuating proportions of social and solitary nests in the population based on population density (Komdeur 1992; Schoepf & Schradin 2012).

The role of kin cooperation and kin competition in the evolution of reproductive skew

The amount of competition within social groups can affect how reproductive opportunities are allocated among group members. The outcome of competition for these reproductive opportunities can lead to societies that are communal, where all individuals share reproduction equally (Danforth 1991; Kukuk & Sage 1994; Gilchrist 2006) or to societies where one female monopolizes all reproduction in the group (Reeve & Keller 2001; Lucas et al. 2011). How these reproductive opportunities are allocated will have consequences for the direct fitness of group members. When group members are not reproductive but remain at the nest to help they are often termed altruistic. The altruism expressed by these individuals can often be explained by kin selection, where the direct fitness opportunities lost by not reproducing are recuperated by helping to raise the offspring of kin (Hamilton 1964a, 1964b). According to Hamilton, genes for altruism will spread if $r_k b > r_o c$, where r_k is the relatedness of the altruist to the kin she helps raise, b is the benefit (the number of additional offspring that are reared because of help), r_o is the relatedness of the altruist to her own kin, and c is the cost of helping (the number of offspring the altruist would have raised on her own).

Within Hamilton's Rule, much empirical research has focussed on calculating r (Bourke 2011; and see Richards et al. 2005, Leadbeater et al. 2011, Rehan et al. 2014 and Gadagkar 2016 for examples where b and c were also calculated). General trends show

that in societies where reproduction is monopolized by a single individual group relatedness is high, increasing indirect fitness benefits to altruists (Jarvis 1981; Chapman et al. 2000; Lucas et al. 2011). In societies where reproduction is partitioned equally among group members, relatedness can vary but is often low (Kukuk & Sage 1994; Danforth et al. 1996; Paxton et al. 1996). This indicates that high relatedness is often accompanied by high levels of reproductive skew. However, several studies have detected unrelated or unexpected patterns or relatedness among social groups (Richards et al. 1995; Queller et al. 2000; Soro et al. 2009; Leadbeater et al. 2010), demonstrating that not all individuals in the group follow the same rules and that direct fitness benefits may play a larger role than originally thought.

An opposing force to kin cooperation is kin competition, where related individuals compete with each other for important resources or reproductive opportunities (Platt & Bever 2009; Van Dyken 2010). West *et al.* (2001, 2002) suggest that Hamilton's Rule should be modified to account for competition among kin by calculating *b* such that $b = B - a(B-c)$ where *c* is the cost of helping as stated in Hamilton's Rule, *B* is the benefit where there is no competition among recipients and *a* is the spatial scale on which competition occurs, from global (competition is spread equally among all individuals in the population, $a = 0$), to local (all competition is occurring within the group, $a=1$). When competition is global, $B=b$ and Hamilton's Rule applies directly, but increasing local competition will in turn decrease the benefits of helping. Kin competition has been shown to override kin selection in related male fig wasps when mating opportunities with females are low (West et al. 2001). Moreover, a recent model and empirical study have

shown that when resources are low and group sizes are small, increased competition among kin leads to increased aggression within the group (Biernaskie & Foster 2016).

A simple behavioural mechanism to reduce competition among kin is through dispersal. By dispersing away from one another, kin reduce *a* by making competition more global and less local. Dispersal as a mechanism to reduce kin competition has been reported in the fig wasp *Platyscapa awekei* (Moore et al. 2006) and the common lizard *Lacerta vivipara* (Lena et al. 1998; Cote & Clobert 2010). In both species, high levels of competition among kin cause increased rates of dispersal when compared to situations of low kin competition. In *L. vivipara*, when kin competition is low, individuals adjusted dispersal rates relative to dispersal risk, dispersing less as risk increased. However when kin competition was high, dispersal rates did not change based on risk, indicating that the drive to reduce competition among kin is strong enough to override environmental risk factors (Cote & Clobert 2010). While dispersal away from relatives reduces competition among them, it in turn leads to the breakup of social groups and a reduction in group size, in its most extreme form leading to solitary individuals across the landscape.

Incorporating the concepts of kin competition, kin cooperation and resource availability, it is possible to generate predictions about group relatedness based on the outcome of competition for reproductive opportunities in social groups. An abundance of time and space for multiple individuals to reproduce will reduce competition among kin and in turn promote kin cooperation leading to a reduction of the number of individuals dispersing away from the nest. If the original group is comprised of kin, as often happens when multiple siblings are reared or laid in the same place, group relatedness will be high and remain that way across the breeding season. If there is only time and space for few

individuals in the nest to reproduce, competition among kin will increase, decreasing kin cooperation. Increasing levels of kin competition will eventually surpass the benefits of kin cooperation and in this situation related individuals should disperse away from one another in an attempt to reduce competition among kin, in turn decreasing overall group relatedness.

Competition and the evolution of sociality in eastern carpenter bees

The eastern carpenter bee, *Xylocopa virginica* is a particularly good model species to understand how competition for resources and reproductive opportunities shape the composition of social groups, as both solitary and social nests can be found in the same nesting aggregation (Gerling & Hermann 1978; Richards 2011). It is therefore possible to quantify what environmental or ecological conditions alter the proportions of solitary and social nests seen in the population. Their life history and nesting preferences may also lead to competition for reproductive opportunities in social nests, as well as for the nests themselves.

Xylocopa virginica overwinter in their natal nests and emerge in spring, presenting females with the choice of cooperating or competing with their nestmates who may or may not also be kin. Previous studies have shown that many females disperse from their natal nest in the spring, either to join already established groups in the population, to excavate their own nests, or to leave the population entirely (Peso & Richards 2010b; Richards & Course 2015). Within social nests females display a dominance hierarchy, where one female monopolizes both foraging and egg laying at any given time (Chapter 4). Subordinate females in social nests are thus non-reproductive.

Until the recent development of microsatellite makers to infer relatedness (Vickruck 2015), we were unable to assess relationships among females in social nests.

In addition, eastern carpenter bees nest in wood and must find and excavate nest tunnels prior to rearing offspring (Velthuis & Gerling 1989). Potential carpenter bee nest sites are non-uniformly distributed in the landscape and are costly to build, making them a valuable resource. As a result, nests are often reused for many generations (Rau 1933; Gerling et al. 1989).

Objectives

Our main objective was to understand the ecological and evolutionary forces that shape sociality in the eastern carpenter bee. We approached this question examining both social group formation and the composition of individuals within social nests. If competition for nest sites (as evidenced by high population density) influences social nesting, we would expect to see more females coming together to share nesting substrate and form social groups when population density is high, and fewer females forming social groups when population density is low. Within social nests dispersal patterns and group relatedness across the season can provide clues as to how competition among kin shapes group membership. If competition among kin is high enough to outweigh the benefits of kin cooperation, we would expect low relatedness among social groups during the reproductive season. If overwintering groups are made up of related individuals, group relatedness should decrease before offspring are provisioned. In contrast, if kin competition is low among social groups, social nests during the reproductive season should be comprised of related individuals, and levels of group relatedness should not

decrease prior to the breeding season. We used detailed behavioural observations and species specific microsatellite markers to observe how group composition changed across the season in two very different years, one of high population density and one of low population density.

Methods

Description of field sites

We studied five nesting aggregations of *Xylocopa virginica*, each one located in a wooden bridge at the Glenridge Quarry Naturalization Site (GQNS), in St. Catharines, Ontario, Canada (43.122, -79.236 decimal degrees). Each bridge was home to 10-22 nests. Bridges were constructed in 2003 and were available for the bees to use as nesting substrate beginning the spring of 2004. Eastern carpenter bees often reuse nests for many years. The term new nest refers to nests that were constructed in the current year, while old nests refers to nests that are being reused from previous years.

X. virginica *colony cycle in southern Ontario*

Eastern carpenter bees are univoltine, producing one brood per year and bees typically overwinter as adults inside natal nests (Gerling & Hermann 1978; Richards & Course 2015). In southern Ontario, emergence takes place in late April or early May and foraging ceases near the beginning of July. *Xylocopa virginica* females have two distinct foraging phases (Richards & Course 2015, Chapter 3). The nestmate provisioning phase (NPP) takes place just after spring emergence and females bring back pollen to the nest to

feed other adult conspecifics rather than to provision brood (Richards & Course 2015). The NPP is also the time when females often disperse away from the nest in which they overwintered. Bees were categorized as resident or transient females based on behavioural observations over the course of the foraging season. Resident females never dispersed and were only ever seen in one nest, while transient females dispersed away from the nest in which they overwintered and were observed in more than one nest. The NPP is followed by the brood provisioning phase (BPP) where pollen brought back to the nest is used to create the large pollen balls on which females lay their eggs. Once brood have been provisioned females remain in the nest to guard developing offspring from predators and parasites. Bees eclose in late-July early-August.

Bee handling and observations during the nestmate and brood provisioning phases

Bees were captured at nest entrances using cup traps. New, unmarked individuals were marked using a unique two paint colour-combination. Females were also measured across the widest part of their heads (head width) to use as a proxy for body size comparisons. At this time the last tarsus of the left mesothoracic leg was removed and placed in chilled 100% redistilled ethanol for genotyping at a later date.

Foraging observations took place for 8h periods (8:00h-16:00h) during the nestmate provisioning phase on days where there was no rain and the temperature was greater than 20 °C. Methods of foraging observations, assigning reproductive strategies, and determining nest status (social or solitary) were done in the same manner as described in Chapter 4.

Winter nest sampling procedures

To quantify the relationships among individuals inside overwintering nests, nineteen nests were destructively sampled in March of 2012. These nests were carefully planed open to expose overwintering bees. All individuals inside nests were measured, marked and had a tarsal sample taken using the same techniques as summer bees described above. Bees from winter nests were then placed into observation nests for the recognition study which took place in Chapter 3.

Genetic analyses and relatedness calculations

DNA extraction and genotyping procedures are described in Chapter 2. In 2012, 189 females from 71 nests were genotyped. In 2013, 101 females from 64 nests were genotyped. Sixteen females were excluded from analyses of relationships in 2012 and 8 in 2013 due to missing data at more than 2 loci.

Relatedness was calculated using the method described by Queller and Goodnight (1989) as implemented in the program Kingroup V2 (Konovalov et al. 2004). Kingroup V2 allowed us to differentiate which pairs of bees within nests were significantly more likely to be full sisters. Hymenoptera are haplodiploid (females are diploid while males are haploid) therefore full sisters inherit one of two maternal alleles and must inherit the single paternal allele. When comparing full sisters, this means that full sisters must share the paternal allele at all loci.

Modelling relatedness distributions

We used a randomization analysis to determine if the number of sisters observed nesting together in the population was different from the number of sisters that would be observed nesting together if females were randomly distributed into nests. We assigned all females marked in either 2012 or 2013 into simulated nests at random. In each sample year, the number of nests as well as the size of the nest (the number of females recorded inside) was replicated exactly as was seen in the population including both solitary and social nests. After females were randomly assorted into nests, we used Kingroup V2 (Konovalov et al. 2004) to determine how many full sister pairs were present in simulated nests, as well as how many simulated nests contained full siblings. We then repeated this procedure 100 times for both the 2012 and 2013 datasets. Simulation results were used to generate empirical distribution functions for the number of nests containing siblings and the number of sisters present for both 2012 and 2013. We then compared our observed values in 2012 and 2013 to the empirical distribution functions to quantify the probability of our observations given the simulated data.

Results

Demographic differences among sampling years

Population densities varied dramatically between the two years of study. At the same study site, 189 females were marked in 2012 and 101 females marked in 2013. Six tertiary females marked in 2012 were recaptured in 2013, three of which remained in the population in as solitary females. There were significantly more females per nest and

proportionally more social nests in 2012 than in 2013 (Table 5.1). Dispersal patterns also differed between years. In 2012, when population density was high, a higher proportion of females disappeared from the population entirely and fewer females were residents. In 2013 more resident females were seen in the population and fewer bees disappeared (Table 5.1).

In 2012 (high population density) nine new nests were excavated while in 2013 (low population density) no new nests were excavated. All new nests were social and contained two females, a primary female and a secondary joining female. In two of these nine nests, no pollen trips were made once nest excavation was complete. None of the new nests were comprised of full sisters (mean r=0.012 ± 0.338).

In 2013 when solitary nests were abundant, 8/26 solitary nests and 2/38 social nests were overrun by ants (*Crematogaster* sp.). Ants were more successful at taking over solitary nests than social nests in 2013 (Fisher's exact P=0.007). No nests were destroyed by ants in 2012.

Nestmate relationships within social nests

The mean proportion of sisters in nests was highest in winter, decreasing in the NPP and was lowest in the BPP, however there were a few overwintering nests which contained no siblings (Figure 5.1a). Within-nest relatedness decreased non-significantly from winter to the BPP (Figure 5.1b). Dispersal and relocation by females resulted in only 30 sisters nesting together (15 pairs, 11% over two years), while 178 sisters (89 pairs, 67% over two years) nested in different nests (Table 5.2). Fifty-eight females (22%) did not have a sibling detected in the population (Table 5.3). The proportions of

females nesting together, apart, or without siblings in the population was the same in both 2012 and 2013 (Table 5.2). On a per nest basis, 86 (85%) social nests contained no sisters, while only 15 (15%) of social nests contained at least 2 sisters (Table 5.2). The proportion of social nests that contained sisters did not differ between 2012 and 2013 (Table 5.2).

Relatedness among females in social nests

Mean within nest relatedness among adult female nestmates during the summer was low (r= 0.125±0.34). This was significantly lower than expected for full sisters (r=0.75, 1 sample t=-18.29, d.f.=101, P<0.0001), but significantly greater than 0 (1 sample t=3.64, d.f.=101, P=0.0002).

Because there were many sets of siblings in the population, females dispersing to new nests may have occasionally ended up with their sisters by chance. Randomization analysis (Figure 2) demonstrated that pairs of sisters nested together no more often than they had been randomly assigned to nests. In 2012, 10 nests contained full siblings. In the randomization, 7/100 simulations produced scenarios in which 10 or more nests contained at least one pair of full sisters, indicating that our result fell within 95% of the distribution (Figure 2a). In 2013, only 4 nests contained full siblings during the summer. The randomization produced 14/100 simulations where four or more nests contained siblings (Figure 2c). The same pattern was observed by nest. In 2012, there were 11 pairs of sisters within nests. Simulation analysis produced 9/100 runs where 11 or more pairs of sisters were found in nests (Figure 2b). In 2013, we found 4 pairs of sisters in nests. Fifteen of one hundred trials generated four or more pairs of siblings (Figure 2d).

Population density and dispersal patterns

The proportion of females relocating to new nests depended on the reproductive strategy employed by the individual female as well as the year. The proportion of transient females versus resident females was similar in both 2012 and 2013 for solitary and primary females (Table 5.3). However in 2012 when population density was high, significantly more transient secondary females were seen in the population than in 2013 when population density was low (Table 5.3). Tertiary females are residents by definition and do not relocate to other nests in the population.

Discussion

Dispersal leads to a reduction of kin competition in social nests

When female carpenter bees become active in the spring, they are first able to assess the level of competition within the nest itself. The theoretical frameworks of Hamilton (1964) and West et al. (2002) suggest that under the right conditions, high levels of kin competition followed by dispersal can lead to social groups comprised of unrelated individuals. Our results also suggest that competition among kin is higher within the nest (local) than among individuals in the population (global).

While winter nests contained higher proportions of sisters than nests during the nestmate or brood provisioning phases, overall levels of within nest relatedness were still lower than expected for full siblings. Relatedness comparisons among females in winter nests suggest that in many cases a single female does not produce all of the offspring in

the nest. This is not surprising given that intense behavioural observations have shown that many primary females do not survive the season and are replaced by other females in the nest (Chapter 4, Richards and Course 2015). Multiple mating has also been observed by females at the field site and decreased relatedness could also be a result of multiple paternity. Finally, it is possible that dispersal takes place in the fall prior to overwintering. However, while females are seen outside of the nest after eclosion, activity levels are very low and females return to the same nest from which they left, making this the least likely cause of lowered relatedness within winter nests (Duff, Vickruck & Richards, in prep.).

Even though relatedness in winter nests was lower than expected if all occupants were full sisters, evidence still exists for competition among kin inside nests. Dispersal has been shown as a mechanism to reduce competition and levels of aggression among kin (Moore et al. 2006; Cote & Clobert 2010) and appears to be the behavioural response shown by eastern carpenter bees. By dispersing away from one another, sisters shift the level of competition from within the nest (local) to within the population (global; West et al. 2002). All nests except one nest in 2013 contained at most one pair of siblings. Overwintering nests start out with varying levels of relatedness but by the brood provisioning phase have at most one pair of siblings present indicating that more dispersal takes place in nests containing larger numbers of siblings. Interestingly, immediately after winter emergence when relatedness is at its highest aggressive interactions are common within nests (Chapter 3). In contrast, during summer when females are provisioning offspring, very little aggression is observed outside the nest or heard inside (frequent buzzing can be heard inside *X. virginica* nests in the spring; J.

Vickruck, pers. obs.). Anecdotally, it appears that there is more conflict in spring nests prior to dispersal than there is in summer nests once competition among kin has been reduced.

Evidence of competition for nest sites

The number of bees present in the population in 2012 was much higher than in 2013, which resulted in significantly more solitary nests in 2013 when there were fewer females vying for space in nests. The number of females per nest during the summer also differed, with more females per nest in 2012, the year of high population density. In addition, new nests were only constructed in 2012 when population density was high. These results suggest that different population densities result in different levels of competition among females for nest sites, and that limited nesting resources facilitate social group formation in *X. virginica.* The pioneering work by Emlen (1982) predicted that increasing constraints on critical resources in the environment would lead to an increase in social nesting. This ecological constraint hypothesis has been demonstrated empirically by variety of taxa including fish, birds and small mammals (Hatchwell & Komdeur 2000; Bergmüller et al. 2005; Schoepf & Schradin 2012).

The result of competition for nest sites suggests that given an abundance of nesting substrate *X. virginica* females would prefer to nest solitarily. Richards (2011) showed that the number of brood per *X. virginica* nest did not vary by group size, meaning that the number of offspring produced per capita actually deceases with increasing group size. Taken together, these two results suggest that solitary nesting may be the most desirable reproductive strategy available to eastern carpenter bees.

Dispersal patterns also provide evidence of competition for reproductive opportunities in social nests. Significantly more females dispersed away from the local population in 2012 when population density was high while more females stayed in their natal nests in 2013 when the population density was low. Solitary nesting was not only higher in 2013 when population density was low, but 40% of solitary females were transients, indicating that dispersal had led to immediate direct fitness benefits. Significantly fewer secondary females dispersed in 2013 when population density was low, providing evidence that smaller group sizes led to lower competition (and therefore less dispersal) within nests. Positive density-dependent dispersal is a well documented response to increased competition and provides further evidence that competition is higher when more females are seen in the population (Waser 1985; Porter & Dooley 1993).

Eastern carpenter bee females should disperse if relocating to a nest will improve their position in the reproductive queue and/or if dispersal will reduce competition among kin. For females who become primary or solitary females in their natal nests, dispersal should be low given that these females will have immediate reproductive opportunities without the associated risks of dispersal. Females who do not become primaries in their natal nest will either become resident, secondary females, or can disperse and attempt to join new nests in the population. Females who are near the bottom of the queue may be better served to disperse and attempt to join new nests where they could establish themselves closer to the front of the line. Indeed, in 2012 when nests were larger (and queues presumably longer) significantly more secondary females were transients than in 2013 when group sizes were smaller and queues shorter. Tertiary females presumably

assess levels of competition within their natal nest only, as they never disperse to other nests in the population.

The winter of 2012/2013 was atypically cold in southern Ontario, Canada and there were several periods where temperatures fell below -25°C, the mean supercooling point (i.e. temperatures lower than this are lethal) of eastern carpenter bees in Niagara (Skandalis et al. 2011). These cold conditions likely contributed to large overwintering losses, and many more dead bees than expected were seen ejected from nests in the early spring of 2013. The large difference in population density from 2012 to 2013 was likely due to the harsh winter conditions bees encountered between the two field seasons.

Potential consequences of allowing dispersing females to join nests

Siblings are clearly dispersing from natal nests, but the question remains as to why a dominant bee would allow another female to join the nest. Allowing new females to join the nest can have consequences for resident females, as many primary females (20.9% in 2012 , 9.7% in 2013) were transients, indicating they had joined the nest and made their way to the top of the reproductive hierarchy, displacing the previous primary female.

In early spring, females are active and make pollen trips, but the pollen brought back to the nest is used to feed other adult females, not for provisioning offspring (Richards & Course 2015) and smaller females feed larger females in the nest (see Chapter 3). This suggests that smaller, potential secondaries may be bringing food 'payments' to dominant females during the spring to gain access to nests as suggested by

Kokko (2002). This pay-to-stay concept has been observed in cichlid fish (Bergmüller et al. 2005; Stiver et al. 2005), as well as birds (Reyer 1984; Dunn et al. 1995).

It is also possible that dispersing females force their way into the nest. While many females were observed trying to gain entry, they were almost never successful. Guarding females are particularly good at prohibiting entry to the nest by using their abdomens to completely block the entrance. On the rare occasion a female does manage to slip past a guard, she was forcibly ejected from the nest (J. Vickruck, pers. observation, chapter 3). This is contrary to Prager (2014) who stated that guards do not prevent entry of non-nestmate conspecifics. This is perhaps the mechanism used by transient females who eventually become primaries in their new nest, as aggressive behaviours are used to establish dominance rankings in carpenter bees (Chapter 3, Hogendoorn and Velthuis 1999).

Finally, it is also possible that there is little cost or potentially even a benefit to allowing other females to join the nest. Significantly more solitary than social nests were destroyed by ants 2013, suggesting social nests may be better at preventing nest userption by *Crematogaster* ants. Parasitism was also higher in solitary nests of the small carpenter bee *Ceratina australensis* and under conditions of extreme parasitism could actually promote social nesting in this species (Rehan et al. 2011).

The evolution of social groups in X. virginica

The finding that social groups are comprised of unrelated individuals is atypical for social insects, but not unexpected under kin selection (Hamilton 1964a; West et al. 2001). In most eusocial societies, non-reproductive helpers at the nest obtain indirect

fitness benefits through shared genes that are identical by descent to the current reproductive. While rarely seen empirically, kin selection theory also predicts that competition among kin can completely override benefits of kin selection. One method to reduce this kin competition is for related individuals to disperse away from one another. Until now, there were no empirical examples of the Hymenoptera employing this strategy in nature. Interestingly, the model generated by Wild and Koykka (2014) suggests that cooperation is actually more likely to evolve when competition is allowed among relatives.

This study examined *Xylocopa virginica* at the northern part of its range, where breeding seasons are the shortest and inbreeding the highest. In the southern portion of the range reproductive seasons may be longer, and Gerling *et al.* (1981) suggest that there may be time for a second brood. Studying eastern carpenter bee populations in the southern portion of their range where breeding season length is longer would provide insights into how breeding season length may impact alter competition both for nest sites and among kin in social nests.

Conclusions

Eastern carpenter bees display a novel form of sociality that is shaped by kin competition within the nest as well as competition for the nest sites themselves. The increased number of solitary nests when population density is low indicates that *X. virginica* females would prefer to nest solitarily, but are forced together by high levels competition for nesting substrate when population density is high. Competition among kin for reproductive opportunities induces dispersal of kin away from one another. These

two competitive forces have led to the evolution of unrelated social groups which display a division of labour. While predicted by the theoretical frameworks of Hamilton (1964) and West *et al.* (2002), this is the first account of this type of social structure in the Hymenoptera. Eastern carpenter bees therefore offer an exciting new vantage point to explore how the evolution of social groups is shaped under high levels of resource and kin competition.

Table 5.1. Evidence for nest site competition between high density (2012) and low density (2013) years. The number of nests refers to the number of active colonies during the season. Number of females per nest was calculated during the brood provisioning phase. All new nests contained two females and are included in the social nest category.

	Year	
Characteristic	**2012 (High density)**	**2013 (Low density)**
Females marked	189	101
Residents	66 (35%)	64 (64%)
Transients	30 (16%)	14 (14%)
Disappeared	80 (42%)	20 (20%)
	X^2= 21.4, d.f.=2, P=0.00002	
Number of nests	71	64
Solitary nests	2 (3%)	26 (41%)
Social nests	69 (97%)	38 (59%)
	X^2= 29.26, d.f.=2, P<0.00001	
New nests	9	0
Females/nest (mean±S.D)	2.55±0.70	1.52±0.73
	Mann Whitney U=1542, P<0.00001	

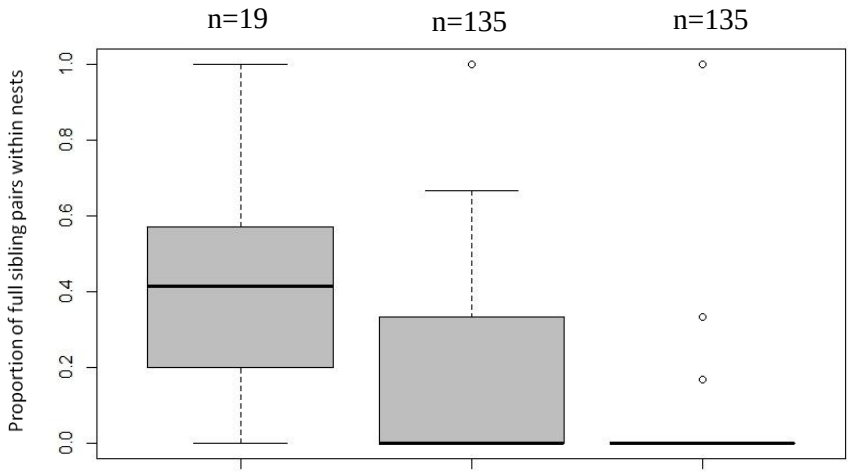
Table 5.2. The location of sisters in low and high competition years by individual and by nest. The proportion of sisters who nested together or apart did not differ over sample year, nor did the proportion of nests which contained sisters.

		Year		
		2012 (High competition)	**2013 (Low competition)**	**Total**
Individual	Nested together	22 (13%)	8 (9%)	30 (11%)
	Nested apart	112 (65%)	66 (71%)	178 (67%)
	No sister in population	39 (23%)	19 (20%)	58 (22%)
	Total Females	**173**	**93**	**266**
		X^2=1.38, d.f=2, P=0.50		
Nest	With sisters	11 (17%)	4 (11%)	15 (15%)
	No sisters	53 (83%)	33 (89%)	86 (85%)
	Total nests	**64**	**37**	**101**
		Fisher's exact, P=0.48		

Table 5.3. The relationship between reproductive strategy and dispersal in both high population density (2012) and low population density (2013) years. Percent dispersed represents the proportion of each reproductive strategy that dispersed in each year. The proportion of solitary and primary females dispersing did not change under different population densities, while the proportion of secondary females dispersing was significantly higher in 2012 when population density was high.

	2012				**2013**				
	Resident	**Transient**	**Total**	**% Dispersed**	**Resident**	**Transient**	**Total**	**% Dispersed**	**Fisher's exact P**
Solitary	2	0	2	0.0%	15	10	25	40.0%	0.13
Primary	34	9	43	20.9%	28	3	31	9.7%	0.33
Secondary	15	21	36	58.3%	11	1	12	8.3%	**0.003**
Tertiary	15	0	15	0.0%	10	0	10	0.0%	NA
Total	**66**	**30**	**96**		**64**	**14**	**78**		

a)



b)

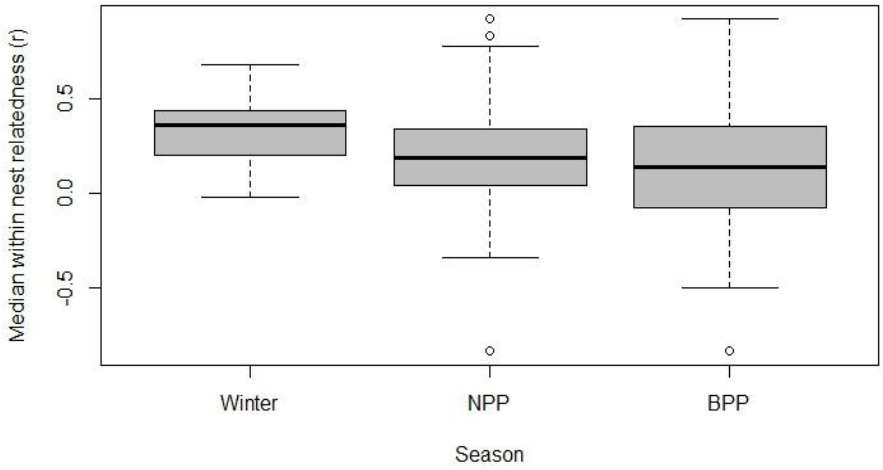


Figure 5.1. Changes in mean nest relatedness from winter to the brood provisioning phase. a) The median proportion of nest mate pairs that were full sisters decreased from winter, through spring to summer (Kruskal-Wallis X^2=13.01, d.f.=2, P=0.001). b) Median within-nest relatedness decreased non-significantly from winter to summer (2-way ordered ANOVA by season and year: $F_{(3,123)}$=1.70, P=0.17). NPP= Nestmate provisioning phase, BPP= Brood provisioning phase.

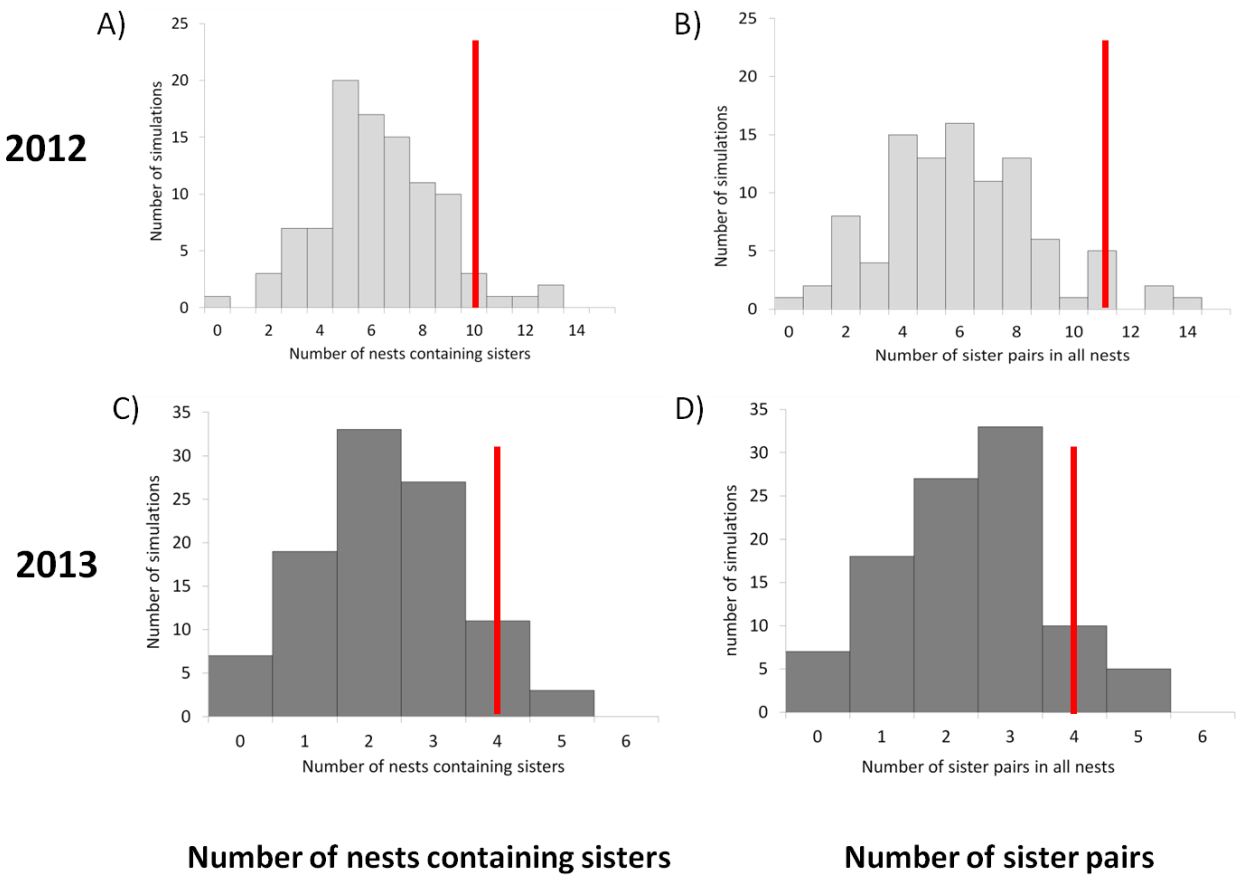


Figure 5.2. Randomization analysis to test departure from the hypothesis that females were randomly distributed among nests. Grey bars are the distribution generated by 100 simulations of random mixing of 71 nests and 189 bees in 2012 and 64 nests and 101 bees in 2013. Vertical red lines indicate the observed value in our populations. A) The randomized number of nests that contained full sisters in 2012. B) The randomized number of overall pairs of sisters in 2012. C) The randomized number of nests that contained full sisters in 2013, D) The randomized number of sister pairs in 2013.

Chapter 6: General Discussion

The ecological and evolutionary pressures that underlie the formation of social groups is undoubtedly one of the most interesting questions in evolutionary biology. This thesis explored the social evolution and molecular ecology of the eastern carpenter bee, *Xylocopa virginica,* from different perspectives, from behaviour inside nests that determines dominance rankings, to how bees in social nests respond to changes in the reproductive queue, the forces facilitating group formation in the first place, and overall population genetic structure. Because eastern carpenter bees can display both solitary and social life histories, consequences and drivers of social versus solitary life can be examined within the same species. This is a powerful tool when studying the evolution of social groups, and it has been argued that obligately social species such as honey bees and many species of ant have passed the 'point of no return', where individuals are fixed in their behavioural roles and under a different type of selection (Wilson & Holldobler 2005).

Adaptability and nesting biology influence overall population structure

In addition to social structure, nesting preferences can impact population structure across the distribution of a species. By nesting almost exclusively in milled lumber, *Xylocopa virginica* is one of the few species of bee that actually benefits from anthropogenic disturbance. Population genetic analysis showed that eastern carpenter bees display a considerable amount of structure across their population, and peripheral populations were distinct from those at the core (Chapter 2). Despite structure among

groups, populations showed generally high levels of genetic diversity and very low levels of inbreeding.

This study was the first to assess the population structure of a native pollinator that is linked to anthropogenic disturbance. The importance of pollination services is widely recognized (Losey & Vaughan 2006; Potts et al. 2010) and the health of native bees has recently received much attention (Goulson et al. 2015; Kerr et al. 2015; Koh et al. 2016). Bumblebees, to which *X. virginica* are morphologically similar, react negatively to anthropogenic disturbance and particularly climate change (Kerr et al. 2015). However, eastern carpenter bees appear able to adapt to constantly changing environmental conditions and are thriving even when nesting in association with humans (Chapter 2). The contrasting results between carpenter and bumble bees highlight the need for species specific population genetic studies to assess pollinator health, as the individual biology of each species will impact how it reacts to anthropogenic disturbance.

Moving forward, understanding which environmental and landscape factors impede gene flow across *X. virginica* populations will further our understanding of the specific elements of disturbance that will have the greatest impact on the population genetic structure. Resistance modeling, which uses detailed landscape data in conjunction with genetic data to understand how environmental features impact gene flow, would provide a more detailed picture of how carpenter bees move through landscapes and would be a logical next step. Traditionally, population genetic studies have focussed on specialist pollinators or those already in decline (Packer et al. 2005; Exeler et al. 2010; Černá et al. 2013). I argue population genetic analyses of a wide range of different

pollinators are needed to truly understand which traits lead some species to be more impacted by disturbance than others.

Food and nest status impacts recognition in X. virginica

Within social groups, how individuals recognize one another can have implications for both group stability and membership. I found that eastern carpenter bees directed more cooperative behaviours and fewer aggressive behaviours towards nestmates but not kin (Chapter 2). In addition, bees returning to the nest with pollen were the recipients of more cooperative behaviours and fewer aggressive behaviours than those that did not. Given that nests during the reproductive period are comprised largely of unrelated bees (Chapter 5), it makes sense that nestmate recognition would be the dominant method of recognition used in eastern carpenter bees.

This study adds to a growing body of literature demonstrating nestmate recognition among social insect species (Breed et al. 1995; Peso & Richards 2010a; Nunes et al. 2011; Breed 2014) and also shows that other contexts such as food rewards influence behavioural interactions among individuals. Cuticular hydrocarbons have been implicated as the underlying mechanism for recognition in a number of insect species (Van Zweden et al. 2010; Nascimento & Nascimento 2012; Lihoreau et al. 2016). To further our understanding of the mechanisms underlying recognition in *X. virginica*, it would be useful to understand the cuticular hydrocarbon profiles of nestmates and non-nestmates, and relatives and non-relatives. If unrelated nestmates have similar hydrocarbon profiles, recognition could be taking place through a binary yes/no group member system. It would also indicate that hydrocarbons may be acquired

environmentally from the nest or surrounding environment. On the other hand, if unrelated nestmates have very different hydrocarbon profiles but are treated as group members, this provides support for individual identification, which is much less common in social insects (Sheehan & Tibbetts 2011). A side project is underway to understand the role of cuticular hydrocarbon profiles in recognition for *X. virginica.*

Alternate reproductive strategies and their influence on dominance hierarchies

Previous work by Richards (2011) defines three reproductive strategies in *X. virginica* females based on wing and mandibular wear, but the rules of how vacated reproductive positions are filled in the queue as well as the role of the tertiary strategy had yet to be quantified. I aimed to understand the rules of nest inheritance throughout the queue as well as the morphological and behavioural differences among reproductive strategies. I demonstrated that eastern carpenter bees form linear dominance hierarchies within social nests (Chapter 4). Successive removal experiments showed that secondary females will always assume the role of new primary in the nest when they reach queue position one, while tertiaries very rarely begin to forage. Females reproducing in the current season (primaries and secondaries) are significantly larger and are less likely to have abdominal fat stores than tertiaries which postpone reproduction until their second spring. By not joining the reproductive queue, non-reproductive tertiary females were often successful at overwintering a second time.

The linear portion of the queue which involves primaries and secondaries is very similar to queues seen in primitively eusocial wasps in the genera *Polistes* and *Liostenogaster* (Cronin & Field 2007; Bridge & Field 2007; Ishikawa et al. 2010),

however tertiary-type females have never been documented in these systems. The act of young adults delaying reproduction and helping at the nest is much more common in vertebrate systems (Woolfenden 1975; Komdeur 1994). This behaviour is particularly unusual for carpenter bees as it means tertiary females are able to double their life expectancy by delaying reproduction, a change that likely entails physiological tradeoffs. Tertiaries were also relatively successful in attaining reproductive opportunities in their second summer, which helps explain how this reproductive strategy is maintained in the population.

To fully explain the reproductive costs and benefits of each reproductive strategy within the population, the next step is to assign actual fitness values to primary, secondary and tertiary positions. This could be accomplished by observing females in the field to assign reproductive strategies, then opening nests at the end of the summer to calculate how many eggs were laid by different females in the nest. Long-term studies of this nature will allow us to determine if different ecological conditions favour different reproductive strategies and would provide evidence that the three reproductive strategies displayed by *X. virginica* are a part of an evolutionarily stable strategy.

Competition leads to unrelated social groups

The behavioural flexibility of *X. virginica* in combination with seasonal variability in population density allowed me to examine ultimate causes of social group formation and division of labour. The formation of social groups was influenced by competition for limiting nesting resources and indicated that carpenter bees would prefer to nest solitarily if nesting substrate was abundant (Chapter 5), supporting Emlen's (1982)

hypothesis of ecological constraint. I also found that while overwintering nests contained siblings, summer reproductive nests contained mostly non-relatives. This suggests that high levels of competition among kin cause sisters to disperse away from one another in an attempt to reduce competition, leading to unrelated social groups.

This finding is novel among the Hymenoptera as competition has never been shown to facilitate the evolution of non-kin based social groups. This alternate route to sociality offers an opportunity to explore alternate routes to sociality. The Xylocopinae are comprised of four Tribes, the *Xylocopini, Ceratinini, Allodapini* and *Manuelini* which display a wide range of life histories, from solitary to eusocial (Rehan & Toth 2015). Both the *Xylocopini* and the *Ceratinini* contain species which are facultatively social, all described members of the *Allodapini* are social and all members of the *Manuelini* are solitary. Understanding how if and how competition has shaped social versus solitary life histories in other species across the subfamily would provide powerful comparative tools to detect overarching factors leading to the evolution of social groups.

The evolution of sociality in eastern carpenter bees

The data from this thesis have allowed me to understand the evolution of sociality in the eastern carpenter bee from a number of different perspectives. Chapter 5 demonstrated the competition for both nesting substrate and among kin has led to the unique form of sociality seen in this species. Chapters 3 and 4 shed light on what influences interactions within social nests, and why both alternate reproductive strategies and dominance hierarchies evolved in social nests. While not explicitly examining the evolution of social groups, chapter 2 demonstrated that flexibility appeared to be a

general trait in this species, as they were able to nest in disturbed areas while even expanding their populations.

Based on the data generated in this thesis, I was able to construct a decision tree for *Xylocopa virginica* females (Figure 6.1). When females emerge after overwintering, many cooperative and aggressive behaviours take place in the nest (Chapter 3). It is at this point that I believe females decide which alternate reproductive strategy to employ. Females who choose to reproduce in the current year fight for a position in the reproductive hierarchy, while those who opt to delay reproduction become tertiary females and move to the end of the reproductive queue. Tertiary females do not disperse, meaning that they make this reproductive decision based on behavioural interactions that take place within nests.

Females who win the competition for reproductive opportunities go to the front of the queue as primary females. In years when competition for nest sites is high this typically means being the primary female in a social nest. In years when competition for nest sites is low, females may also become solitary. Individuals that are not at queue position one have several options: stay in the natal nest at a lower queue position, disperse and join a nest locally, excavate a new nest or disperse away from the population entirely. When competition for nest sites is high, more females disperse away from the population and also join new nests. High levels of competition for nest sites also increases the number of new nests that are constructed in the population. In contrast, when competition for nest sites is low, dispersing females may become solitary, either by occupying empty nests, or displacing the current resident.

Competition for reproductive opportunities determines how many bees disperse

from the natal nest, but competition among kin within the nest dictates which individuals remain and which disperse. Competition among kin for reproductive opportunities within the nest is always high, and related individuals disperse away from one another in an attempt to reduce kin competition. For *X. virginica*, it appears that the cost of helping is greater than the cost of dispersing and attempting to join a new nest. The dispersal of related females away from the natal nest occurs when population density is high and low, suggesting that kin competition is a selective force inside all social nests.

In eastern carpenter bees, sociality appears to be driven by a constant push and pull of competition for nest sites driving individuals together into social nests, and the avoidance of kin competition increasing dispersal and forcing individuals apart (Figure 6.2). High levels of competition for nesting substrate therefore lead to a higher relative number of social nests in the population as competition for nest sites increases. Discovering the unique way in which competition shapes social groups in *X. virginica* is an extremely valuable contribution to sociobiology, as it offers a rare empirical example that has been predicted by theory for decades. Sociality in eastern carpenter bees thus offers a unique opportunity to empirically study the evolution of social groups from a new perspective, one where competition is important above all. I believe this is only the beginning of our understanding of this fascinating species and further investigation will yield more critical insights into our understanding of social evolution.

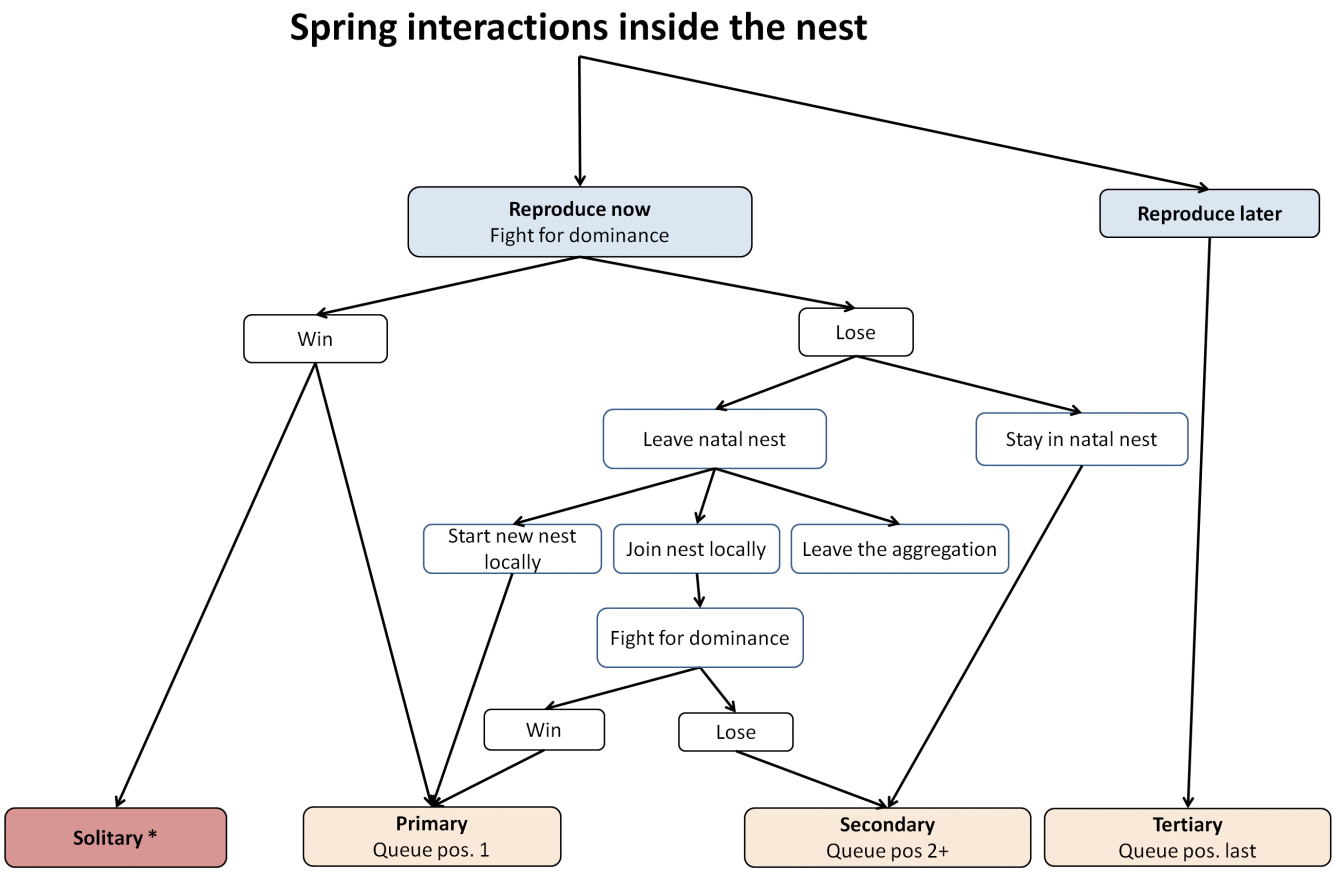


Figure 6.1. Decision tree for *Xylocopa virginica* females in spring. Spring interactions in the nest determine whether females breed in the current reproductive year, or delay reproduction until the following year. * Females may also become solitary by dispersing away from the natal nest in years of low population density.

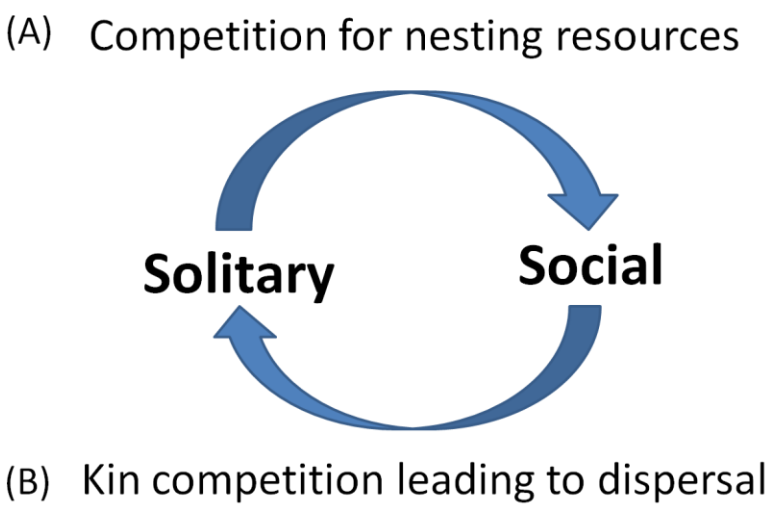


Figure 6.2. Facultative sociality in *Xylocopa virginica* as maintained through competition for nesting sites and kin competition. (A) External competition for nesting sites influences solitary versus social nesting. Low population density leads to decreased competition for nesting resources and increased numbers of solitary nests in the population. (B) Competition within the nest among kin for reproductive opportunities leads to dispersal away from the natal nest, where competition then takes place outside of the nest again.

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Appendix: Development of sixteen novel microsatellite markers for the eastern carpenter bee, *Xylocopa virginica* (Hymenoptera: Apidae), through paired-end Illumina sequencing

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Abstract

Sixteen novel polymorphic microsatellite loci were developed and characterized for the eastern carpenter bee, *Xylocopa virginica* (Linnaeus 1771) using paired end Illumina shotgun sequencing. The number of alleles per locus ranged from 2-15 (mean 8). None of the loci tested showed deviations from Hardy-Weinberg equilibrium or signs of linkage disequilibruim after Bonferroni correction. These variable loci will be used to assess the population structure of this eastern North American pollinator on multiple scales, to understand how gene flow and population structure are modified over fragmented landscapes, and to observe the potential effects of range shifts due to climate change.

Body

Global native bee declines have become of greater concern in the past decade due to changes in land use, pesticide practicees and climate change, unveiling how little we know about native bee fauna (Potts et al. 2010). The eastern carpenter bee is a large, effective generalist pollinator found throughout eastern North America. Their large home ranges (approximately 1 km), also allow pollination of flowers over large distances. Females often nest in aggregations that persist for several years. Prior to European colonization, *X. virginica* nested primarily in newly fallen trees or appropriate horizontal branches, however they now nest almost exclusively in artificial structures. This strong link to humans, and their value as large generalist pollinators makes carpenter bees particularly interesting from a conservation standpoint, as human land use may now be affecting carpenter bee population structure and range expansion. To date there are no suitable molecular marker available to study the population structure of this species.

Genomic DNA was extracted from one metathoracic leg of two female bees collected in St. Catharines, ON using the DNeasy blood and tissue kit (Qiagen). Standard protocol was used with the one additional step: after incubating tissue in lysis buffer and proteinase K, the supernatant was vortexed and transferred to a new microcentrofuge tube to prevent bits of macerated exoskeleton from clogging the spin column. Samples were prepared for Illumina sequencing as per Nunziata et al (2013). The resulting paired-end sequences were then analyzed using PAL_FINDER_v0.02.03 (Castoe et al. 2012) to locate reads containing microsatellite repeats. Primer3 (Untergasser et al. 2012), was then used to generate primers for the remaining paired-end reads. Only primers that amplified a single locus were considered for further testing. Forty-eight primer pairs were chosen

for initial screening with ten unrelated individuals. Of the initial 48 loci tested, 17 were polymorphic and easy to amplify. Eleven additional individuals were then screened at the 17 polymorphic loci. Forward primers contained M13 tails on the 5' end to add a flourescent probe (Schuelke 2000). Loci were amplified individually in 20 µL reactions containing 40-70 ng genomic DNA, 1 U Standard Taq (New England BioLabs), 1x Thermo Buffer (New England BioLabs), 0.2 mM dNTPs, 0.2 µM FAM labelled M13 primer, 0.02 µM forward primer and 0.2 µM reverse primer. PCR conditions were the same for all loci, and PCR reactions were run on a LabNet Multigene Mini (Mandel) at 95°C for 5 min, 20 cycles of 95°C for 30 sec, 65°C for 30 sec (decreasing by 0.5°C per cycle), 72°C for 30 sec, followed by another 20 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec. Labelled PCR products were run on a 3730xl DNA Analyzer (Applied Biosystems) and genotyped using Genemapper v 3.5 (Applied Biosystems). Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium were tested in GENEPOP v4.2 (Raymond & Rousset 1995).

The number of alleles per locus ranged from 2-15 (mean 8 ± 3.8) with observed and expected heterozygosity ranging from 0.273-0.833 (mean 0.67) and 0.398-0.907 (mean 0.735) respectively (Table 1). After Bonferroni correction, none of the loci exhibited deviations from HWE or showed signs of linkage disequilibrium. The markers developed in this study will be used to assess levels of gene flow and population structure of *X. virginica* across its range.

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Table 1. Primer sequences and locus characteristics for 16 microsatellite loci for *X. virginica* screened on 21 individuals from eastern North America.

Locus	Primer sequence 5'-3'	Repeat Motif	*k*	Allele Size Range [a]	H_o	H_e	HWE P
XV1	F: CGAACATGGTAAGAATCTCTCTCTCC* R: GTGGACAACGTTGAAATGCG	AGAGC	10	255-300	0.762	0.876	0.039
XV3	F: ACCTGGATGGCGGAGAGC* R: GTTGGCGGTGGGTGTACG	TTCC	11	229-261	0.750	0.827	0.386
XV7	F: GCTCGACGTACCCTTGCG* R: GTGGCAGTGACGTGGTGG	TGCC	8	318-362	0.833	0.765	0.295
XV9	F: ACTCTATTATTCTACATTAGTACGGTTCGC* R: TTCGATTTCTGGCCTCTTCG	TGCG	4	199-211	0.571	0.712	0.495
XV10	F: GGAAATCGGAGGACGAACC* R: AACCCTGCTTCCTCCTTATGC	TGCC	6	207-227	0.714	0.747	0.939
XV12	F: CCTATTGATGAGATGATTTCTATACTATGC* R: CCATACACTGTGCCAAACG	AAAG	4	216-228	0.273	0.398	0.627
XV14	F: AAGACCCGTTACCCTTTCCC* R: CGCGTGTAACCAAACGTCC	ACTG	2	333-337	0.286	0.398	0.330
XV23	F: AGACGAGAGCGACGAGGG* R: GTATGCACATTGCACACGC	TTC	12	395-427	0.823	0.887	0.931
XV24	F: CACAACCACAGCCACAGTCG* R: GCCACCTGTCCAAGACTGC	TGC	6	206-221	0.762	0.699	0.940
XV27	F: GAACAAGAGGACGGCAGAGG* R: CCAGCACTGCAGACAGTGTACC	TGC	9	239-265	0.619	0.877	0.011
XV28	F: CCGAGCTTCTGCTCTTCTGC* R: CCTACCACCGTCCGATCTCC	TTC	12	267-303	0.813	0.907	0.142
XV29	F: CTTCGCACCTCTTTCAACCG* R: GAGATTCTTCTCCGCGATCC	TTC	6	251-268	0.762	0.753	0.750

XV30	F: TTGATATAGCGCCGACCTCC* R: TCCTCTCGCCAAGTCTCCC	ACC	4	306-319	0.612	0.599	0.089
XV39	F: CGGCGTAGTGGTGGTACTGG* R: GCGTTTCCTTCTCTTTCAGAGC	ACC	15	233-267	0.714	0.801	0.967
XV42	F: CAACGAATACAAACACCAGGTAGG* R: AACCTGCATTCCTTGATACGG	TGC	6	467-481	0.684	0.720	0.932
XV43	F: AGATACACAAGGAGAAGAAGGCG* R: CGAGAGAGTCGAGGGAACG	TCG	13	218-236	0.714	0.787	0.627

***** Indicates M13 tail.

[a] Fragment lengths include and additional 18bp from M13 primer.